Journal of Supramolecular Structure 9:489–496 (1978)
Transmembrane Signaling 411–418

Increase in the Affinity of the Uridine Phosphorylation System for ATP After Serum or Insulin Activation of 3T3 Fibroblasts

Gerald J. Goldenberg and Wilfred D. Stein

Institute of Life Sciences, Section of Biophysics, Hebrew University, Jerusalem, Israel

The stimulation of uridine uptake, brought about by the addition of serum or insulin to quiescent 3T3 fibroblasts, is associated in the half-saturation concentration of the uridine phosphorylating system for the substrate ATP, with relatively little change in the maximum uptake or in the affinity for uridine. In stimulated cells the Km towards ATP fell in the range 0.053–0.187 mM, while V max was 34 to 52 pmoles/10^6 cells/min. In quiescent cells these values were 2.89–4.22 mM and 74.5–126 pmoles/10^6 cells/min, respectively. No difference was found, however, between the Km's for ATP when phosphorylation of uridine was determined using cell-free extracts prepared from either quiescent cells or from stimulated cells.

Key words: fibroblasts, uridine, uptake, quiescent, serum, insulin

Cells maintained in culture may be brought into a quiescent state by transfer to a suboptimal medium and subsequently activated to proliferate by addition of a complete medium [1]. An early event during such activation with serum or insulin is the stimulation of uridine uptake [2]. Recent work has shown that it is not uridine transport but rather the subsequent trapping of uridine within the cell that is so enhanced [3, 4]. This trapping of uridine intracellularly into acid-soluble pools represents phosphorylation as has been shown by thin-layer chromatography of such trichloroacetic acid (TCA) extracts [5].

Uridine kinase, the first enzyme in the pathway of uridine phosphorylation in 3T3 cells, utilizes both ATP and uridine in a bisubstrate reaction [6]. Thus, theoretically, stimulation of uridine phosphorylation can be seen as an enhanced activity of the system in response to uridine, ATP, or to both of these substrates. To ascertain which of these modes is employed in the activation of uridine uptake, we undertook separate kinetic

G.J.G. is on sabbatical leave from the Manitoba Institute of Cell Biology and Department of Medicine, University of Manitoba, 700 Bannatyne Avenue, Winnipeg, Manitoba R3E OV9, Canada. W.D.S. is now at the Department of Biochemistry, University of Sussex, Falmer, Brighton, BN1 9QG, England.

Received March 15, 1978; accepted July 7, 1978.

0091-7419/78/0904-0489$01.70

analyses of the interaction of each substrate with the uridine phosphorylating system. In the present study we report an analysis of the trapping of uridine by 3T3 fibroblasts in situ where we have varied separately either the uridine or the ATP levels within the cells. We show that the stimulation of uridine uptake as a result of serum or insulin activation is apparently associated with an increase in the half-saturation concentration of the uridine phosphorylating system for the substrate ATP, with relatively little change in the maximum velocity of uptake or in the affinity for uridine.

MATERIALS AND METHODS

Fibroblasts, cultured in 25-cm plastic petri dishes (Nunc, Copenhagen) in Dulbecco's minimal essential medium (MEM) and supplemented with 10% dialyzed fetal calf serum (FCS), were incubated at 37°C in 5% CO_2 and humidified air. Cells were rendered quiescent by transferring them to a suboptimal medium consisting of 5% FCS in MEM for 10 days and in 0.25% FCS in MEM for 16 hours. An experiment was initiated by replacing the suboptimal medium with approximately 1 ml of either 10% FCS in MEM (serum-activated cells) or 10% phosphate-buffered saline (PBS) in MEM (quiescent cells) and incubating the cells at 37°C for 45–50 minutes.

For measurement of uptake, activation or sham-activation was terminated by replacement with approximately 1 ml of either 10% FCS in PBS (activated cells) or PBS (quiescent cells) containing ^{3}H-uridine at a final concentration of 2 or 4 μCi/ml and cold uridine at a concentration of 5–500 μM. The cells were maintained at 20°C for 5–60 minutes, and incubation terminated by washing five times with approximately 1 ml of ice-cold PBS, and the radioactivity extracted in 5% TCA at 4°C for 20 minutes. Under these conditions the equilibrium level for free uridine was calculated to be 200 cpm/dish, using the cell number per dish, the radioactive counts associated with a known volume of external medium, and a cell volume of 5 pl calculated from the equilibrium distribution level of 3-O-methyl glucose. For those experiments in which the level of intracellular ATP was to be altered, after activation or sham-activation, the medium was replaced with approximately 1 ml of either 10% FCS in PBS (activated cells) or PBS (quiescent cells), both solutions containing various concentrations of 2-deoxyglucose up to 100 mM [7] and the cells being incubated at 37°C for 15 or 30 minutes. Where it was merely the intracellular ATP level that was to be determined, the incubation was terminated at this stage and the ATP extracted in 1 ml of boiling water, the temperature being subsequently maintained at approximately 80°C for 15 minutes. The ATP concentration was determined by the luciferase assay [8]. The intracellular ATP concentration was calculated using the cell number (10^5 cells/dish) and the cell volume (5 pl).

In other experiments, the intracellular ATP concentration and rate of uridine uptake were simultaneously determined in quiescent and serum-activated 3T3 fibroblasts following treatment of the cells with 2-deoxyglucose. The cells were prepared and treated with 2-deoxyglucose (for 15 minutes) as above, the medium was changed to either 10% FCS in PBS for activated cells or PBS for quiescent cells, containing in both cases the appropriate concentration of 2-deoxyglucose and in addition 2 μCi/ml of ^{3}H-uridine and 50 μM unlabeled uridine. The cells were then incubated at 20°C for approximately 5–25 minutes. Uptake of uridine was terminated by washing five times with approximately 1 ml of ice cold PBS. The cell contents were extracted in 1 ml of boiling water and both ATP and radioactivity were determined.

In some experiments 3T3 cells were treated with insulin, 500 ng/ml (24.3 IU/mg) for 50 minutes, while control quiescent cells were not exposed to insulin, using the same conditions of 2-deoxyglucose treatment described above for serum activation.

The velocity of uridine phosphorylation was measured in vitro [9] using enzyme extracts from quiescent or serum-activated 3T3 fibroblasts treated as described above. After activation or sham-activation, the cells were scraped off the dishes with a rubber spatula, suspended in the residual medium, and the cell membranes ruptured by repeated freeze-thawing (7 X) in liquid nitrogen. The enzyme assay was performed using 30 μl of enzyme solution, 10 μl of the appropriate concentration of ATP with equimolar $MgCl_2$, and 60 μl of 150 mM Tris buffer, pH 7.4, containing 50 μM uridine and 44 μCi/ml of ^{3}H-uridine. Incubation was carried out at 37°C for 40 minutes and the reaction stopped in boiling water for 3 minutes. Radioactivity incorporated into nucleotide was determined by spotting 40 μl of the reaction mixture on Whatman DE 81 ion-exchange filter disks, drying under a hot lamp, and washing with approximately 100 ml of 1 mM ammonium formate to remove nonphosphorylated uridine. Total radioactivity on the disk was determined by spotting 5 μl of the mixture and drying, but not washing away, unbound radioactivity. The results were calculated as percentage of total radioactivity present, that is, incorporated into nucleotide.

RESULTS

The time course of uridine uptake by quiescent and serum-activated 3T3 fibroblasts at several uridine concentrations is shown in Figure 1A. These curves are essentially linear over the time period studied, and the slopes of such lines give the velocity of trapping of uridine, since the cpm/dish observed at all points after 10 minutes exceeds the value of 200 cpm/dish estimated for uridine transport at equilibrium, and uridine transport occurs on a facilitated diffusion system [5]. Therefore, we are justified in taking the slopes of such curves as a measure of the rate of in situ phosphorylation.

Figure 1B shows a Lineweaver–Burk plot of the trapping of uridine by quiescent and serum-activated 3T3 fibroblasts as a function of the extracellular uridine concentration. Linear regression analysis of these plots gave values (mean ± SE) of K_m = 7.3 ± 1.3 μM and a V_{max} = 1.56 ± 0.24 pmoles/min/dish for quiescent cells and a K_m = 9.2 ± 1.9 μM and a V_{max} = 3.62 ± 0.67 pmoles/min/dish for serum-activated cells. Thus serum-activation produces more than a 2-fold increase in V_{max} without a significant change in K_m, when uridine is the varying substrate. (We recognize that the pertinent concentration of uridine is its intracellular value rather than extracellular levels used in Figure 1B. Unpublished calculations, using the measured values of the kinetic parameters for uridine transport and phosphorylation, show that for the data in Figure 1B, the intracellular concentration is only 20% less than the extracellular concentration at the K_m values [7]).

To analyze the kinetics of uridine phosphorylation in situ as a function of ATP concentration, it was necessary to set the intracellular level of ATP by appropriate manipulation of the cells. This consisted of incubation of both quiescent and serum-activated 3T3 fibroblasts in different concentrations of 2-deoxyglucose [8] and resulted in a systematic reduction of ATP concentration as demonstrated in Figure 2. It is important to note that both the basal level of ATP and the level of intracellular ATP reached at any level of 2-deoxyglucose administered to the cell, were essentially identical in quiescent and serum-activated cells. The intracellular ATP concentration (mean ± SE, 16 determinations) for cells not treated with 2-deoxyglucose, was 1.31 ± 0.07 mM for quiescent cells and 1.33 ± 0.09 mM for activated cells.

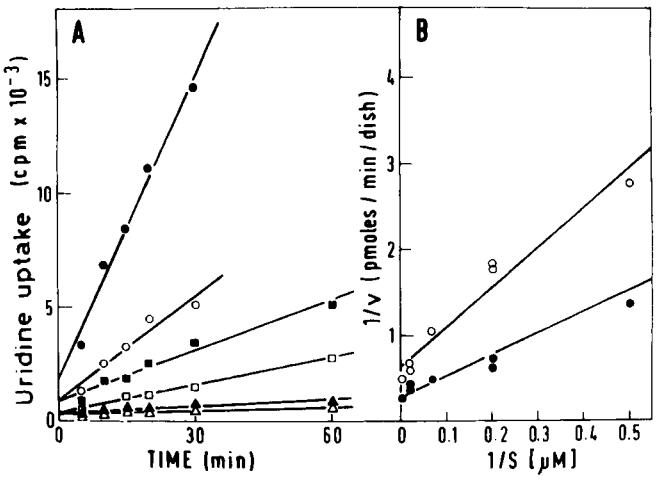

Fig. 1A. Time course of uptake of ^{3}H-uridine by quiescent and serum-activated 3T3 fibroblasts in vitro. Uridine at a concentration of 5 μM (○, ●), 50 μM (□, ■), or 500 μM (△, ▲): open symbols are quiescent cells, solid symbols are serum-activated cells.

Fig. 1B. Lineweaver–Burk plot of the uptake of ^{3}H-uridine by 3T3 fibroblasts in a quiescent state (○) and following serum activation (●). The reciprocal of the uptake velocity in pmoles/min/dish was plotted against the reciprocal μM concentration of uridine. Uptake velocities were determined from linear regression analysis of time courses such as those illustrated in Figure 1A, taking into account the appropriate specific activity.

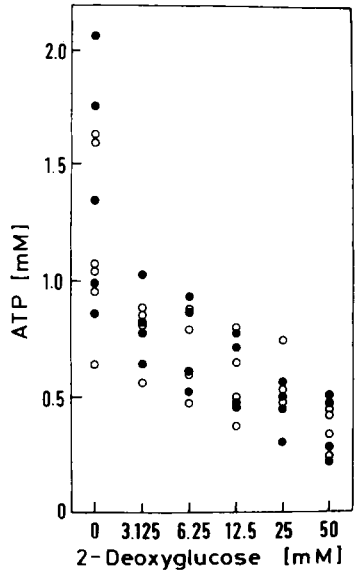

Fig. 2. Intracellular ATP concentration in quiescent (○) and serum-activated (●) 3T3 fibroblasts. Activated and quiescent cells were prepared as described in the text.

Using the 2-deoxyglucose technique to alter intracellular ATP concentration, a kinetic analysis of uridine phosphorylation was performed (Fig. 3). We measured in each case, on the same dish, the intracellular ATP concentration and the amount of labeled uridine that had accumulated within the cells. The mean ATP concentration in quiescent and serum-activated cells as a function of 2-deoxyglucose concentration is presented in Figure 3A. At each concentration of 2-deoxyglucose the ATP level did not change consistently after the first 5 minutes of uridine uptake (in 24 time courses the ATP level increased in seven, decreased in eight, and was not altered in nine cases), and therefore the points in Figure 3A depict the mean (± SE) intracellular ATP concentration during the time in which uridine was being accumulated. The ATP levels in both quiescent and activated cells were lower than in cells which had not been exposed to uridine (compare Figs. 2 and 3A). For the cells that were exposed to uridine but not to 2-deoxyglucose, the ATP levels were now lower in activated than quiescent cells.

Representative time courses of uridine uptake from the same experiment as that of Figure 3A are shown in Figure 3B. From such time plots we can generate a kinetic analysis of the rate of uridine phosphorylation as a function of the intracellular ATP concentration, measured on the same sample, at a uridine concentration 5–7 times higher than the measured K_m for uridine. The data are illustrated in Figure 4A. A computer analysis of these in situ observations yielded the following values of the kinetic parameters on fitting

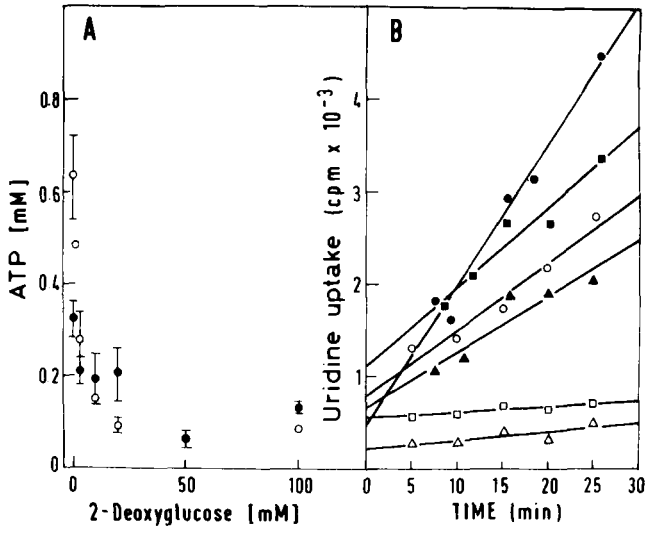

Fig. 3A. ATP activity was measured and the data plotted for quiescent (○) and serum-activated (●) 3T3 fibroblasts as a function of 2-deoxyglucose concentration for cells taking up uridine. Each point is the mean (± SE) of 5 determinations.

Fig. 3B. Representative series of time courses of uridine uptake by the same cells shown in Figure 3A treated with 0 mM (○,●), 9 mM (□, ■), or 100 mM (△, ▲) 2-deoxyglucose; open symbols are quiescent cells, solid symbols are serum-activated cells. The lines were obtained by linear regression analysis.

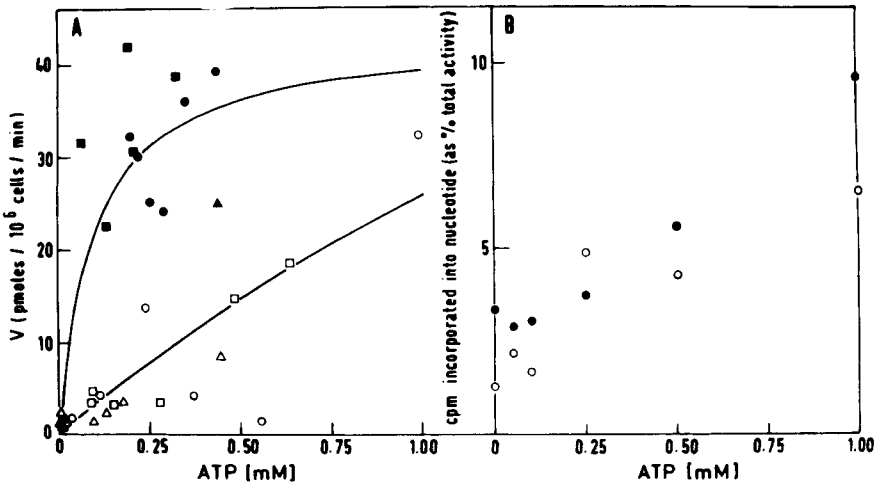

Fig. 4A. Velocity of uridine phosphorylation in activated and quiescent 3T3 fibroblasts as a function of intracellular ATP concentration. The velocities were obtained from linear regression lines such as those shown in Figure 3B and the ATP measurements, obtained as in Figure 3A. The open symbols are quiescent cells, the closed symbols activated cells, circles and squares being separate experiments with serum as activator. The triangles represent an experiment in which 3T3 cells were treated with insulin (▲) or not exposed to insulin (△) using the same conditions of 2-deoxyglucose treatment described for serum-activation in Figure 3. The lines drawn were calculated from the Michaelis–Menten equation using parameters obtained by a nonlinear least squares analysis (computer program NONLSQ, Hebrew University).

Fig. 4B. Velocity of uridine phosphorylation using enzyme extracts from quiescent (○) or serum-activated (●) 3T3 fibroblasts treated as described in the caption for Figure 1A.

these to the Michaelis–Menten equation, giving the solid lines drawn on Figure 4A (we record, in brackets, the range of values within which with a confidence of 95% the derived parameters will fall): i) In activated cells the K_m was 0.097 mM (range 0.053–0.187), while the V_{max} was 43.1 pmoles/10^6 cells/min (range 34.1–52.3). ii) In quiescent cells, the K_m was 2.91 mM (range 2.89–4.22) while the V_{max} was 100 pmoles/10^6 cells/min (range 74.5–126).

An attempt to repeat these observations with enzyme preparations extracted from quiescent and serum-activated cells was undertaken. A kinetic analysis using ATP as substrate revealed no difference in K_m for uridine phosphorylation between enzyme extracts from quiescent and activated cells, and in both cases, the K_m approximated that obtained with quiescent cells in situ (Fig. 4B).

DISCUSSION

Although we can readily reproduce the phenomenon of the activation of uridine uptake (Fig. 1), the data on intracellular ATP levels show that the difference between activated and control cells is not due to any difference in their intracellular ATP concentrations. These data are consistent with earlier reports comparing the nucleotide levels in growing and density-inhibited 3T3 cells [10]. In addition, the response of quiescent and activated cells to various levels of 2-deoxyglucose in the absence of uridine appears

identical (Fig. 2); that is, activation does not enhance the capacity of the cells to maintain a higher level of ATP when such levels are reduced by treatment with deoxyglucose.

Indeed, when cells depleted of ATP by 2-deoxyglucose treatment are then presented with 50 μM uridine, the entry of this nucleoside results in a further depletion of the intracellular ATP levels which can be very marked (compare Figs. 2 and 3A) and is greater in serum-activated than in quiescent cells. The drop in intracellular level is what might be expected from the amount of uridine taken up by the cells. As we proceed to show, in Figure 4A, the rate of uridine uptake in activated cells is 34–52 pmoles/10^6 cells/min. We find that 10^6 cells have an intracellular water space of 5 μl, so the molar concentration of uridine (and its derivatives) will increase by some 7–10 μM per minute. Each uridine molecule that enters will consume three molecules of ATP and be converted to the triphosphate so that a concentration drop of approximately 21–30 μM ATP per minute is to be expected. Consequently, at the end of 25 minutes of incubation with 50 μM uridine (depicted in Figure 3B) we might expect a concentration decrement of 500–750 μM ATP. The ATP concentration apparently drops (compare Figs. 2 and 3A, values for zero 2-deoxyglucose) about 1,000 μM, but the error in this value is very large. In addition, some of the uridine counts are incorporated into acid-insoluble materials (nucleic acids) and these are not measured in Figure 4A. But in unpublished experiments, we have seldom found this to be more than 20% of the counts found in the acid extract.

The fall in ATP level in quiescent cells is not as large but then the uridine uptake is only half as great (Fig. 1B). When the ATP level is already reduced by 2-deoxyglucose treatment, the further reductions brought about during uptake of uridine are quite predictable, as similar calculations show. What is perhaps surprising is that we could see no consistent fall in ATP level during the course of the uridine uptake. We assume that this may be due to the imprecision inherent in a single-point ATP assay.

The fall in ATP level that we observe leads to a serious experimental difficulty; we have not been able to obtain cells with ATP levels higher than 0.6 mM in the case of activated cells taking up uridine. Thus we have not been able to extend the ATP dependence curve to higher ATP concentrations. Nevertheless, the data of Figure 4A do enable us to make some comments as to the mechanism of activation of uridine uptake.

Although the data of Figure 4A show a good deal of scatter, the rate of uridine phosphorylation at any ATP concentration above 0.1 mM is seen to be substantially greater for the activated than for the quiescent cells. This difference, however, diminished with increasing ATP concentration. Hence, the K_m values derived from the data will be different for the two situations. Indeed, the K_m difference between activated and quiescent cells is so great (some 30-fold by the computer analysis) that, within the range of ATP concentrations studied, uridine phosphorylation in serum-activated cells is almost everywhere close to saturation, whereas in quiescent cells the system is well below saturation. This is so for each of the three separate experiments in Figure 4A, as well as for all the pooled data.

Although we would not wish to claim great reliability for the K_m values obtained by the computer analysis (0.053–0.187 mM for serum-activated cells, and 2.89–4.22 mM for the control cells, giving the range of the 95% confidence intervals), this analysis clearly suggests that the effect of serum activation is to enhance substantially the apparent affinity of the uridine phosphorylating system for the substrate ATP. Such an enhancement of affinity for ATP would account also for the apparent increase in maximum velocity when uridine is the varying substrate, since these experiments (Fig. 1) were done at a constant level of ATP (approximately 0.5 mM, see Fig. 3A) where the system was not saturated in the quiescent cells.

However, using enzyme extracts in vitro, we have been unable (Fig. 4B) to demonstrate the enhanced affinity of the uridine phosphorylation system for ATP, which characterizes the activated state in situ. Possibly in the extraction we have diluted the enzyme or diluted out some essential effector needed to demonstrate the enhanced affinity of the in situ condition. Alternatively, preservation of anatomical structure might be essential in order to demonstrate activation.

We are aware, too, of the possibility that changes in numerous other cell constituents may be brought about by treatment with 2-deoxyglucose directly, or secondarily as a result of the depletion of intracellular ATP. These other cell constituents may be regulators of the activity of uridine phosphorylation. But, if so, Figure 4A suggests that their role is likely to be as allosteric modifiers of the enzyme activity, allowing the full activity of the enzyme to be achieved in serum-activated cells in the face of a low concentration of intracellular ATP. Thus, the effect of these postulated modifiers is to bring about the apparent increase in affinity of the enzyme system for ATP. Our conclusion remains that the activation of uridine uptake brought about by serum is operationally an apparent enhancement of the affinity of the uridine phosphorylation system for ATP.

Activation of uridine uptake thus results from an increase in the apparent affinity for the substrate ATP in the in situ uridine phosphorylation system. The apparent 2–3-fold increase in V_{max}, seen when uridine is the variable substrate (Fig. 1B), may readily be accounted for by the fact that such measurements were performed at ATP concentrations less than saturating for quiescent cells, but well above the K_m for activated cells (see Fig. 4A). Indeed, the V_{max} derived by computer analysis of data presented in Figure 4A, where both ATP and uridine are at saturating levels, is slightly higher for quiescent than for activated cells, a reversal of the situation found at the normal ATP levels of these cells.

The possible biological relevance of the change in K_m for ATP may be to ensure that uridine uptake can occur at a rapid rate despite the drop in intracellular ATP concentration that we find occurring when activated cells take up uridine. The mechanism of this higher affinity for ATP in activated cells remains to be established. It may represent either a direct allosteric change in uridine kinase (the rate-limiting step of the uridine phosphorylation system) or else a change in the intracellular concentration of some effector of the system.

ACKNOWLEDGMENTS

We thank Osnat Bibi and Dvorah Ish-Shalom for excellent technical assistance, Michal Razin for assistance with data analysis, and Ruth Koren and Esthi Shohami for their interest and constructive discussions. Financial support by the US NCI No. 1CP 43307 and the National Cancer Institute of Canada is greatly appreciated.

REFERENCES

1. Pardee AB: Proc Natl Acad Sci USA 71:1286, 1974.
2. De Asua J, Rozengurt E, Dulbecco R: Proc Natl Acad Sci USA 71:96, 1974.
3. Rozengurt E, Stein WD, Wigglesworth N: Nature (London) 267:442, 1977.
4. Koren R, Shohami E, Bibi O, Stein WD: FEBS Lett 86:71, 1978.
5. Rozengurt E, Stein WD: Biochim Biophys Acta 464:417, 1977.
6. Anderson EP: In Boyer PD (ed): "The Enzymes." London: Academic, 1973, vol 9, p 49.
7. Renner ED, Plagemann PGW, Bernlohr RW: J Biol Chem 247:5765, 1972.
8. Strehler BL, Totter JR: Methods Biochem Anal 1:341, 1954.
9. Reichard P, Skold O: In Colowick SP, and Kaplan NO (eds): "Methods in Enzymology." New York: Academic Press, 1963, vol 6, p 194.
10. Weber MJ, Edlin G: J Biol Chem 246:1828, 1971.
11. Heichel O, Ish-Shalom D, Koren R, Stein WD: (Submitted for publication).