Does the Bifunctional Uridylate Synthase Channel Orotidine 5′-Phosphate? Kinetics of Orotate Phosphoribosyltransferase and Orotidylate Decarboxylase Activities Fit a Noninteracting Sites Model†

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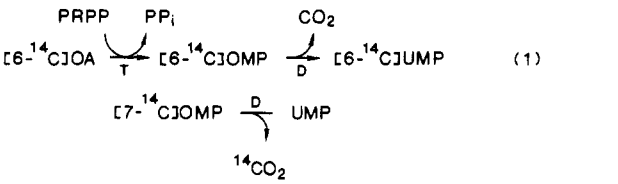
ABSTRACT: Uridylate synthase is a bifunctional protein that first forms orotidine 5′-phosphate (OMP) from orotate via its orotate phosphoribosyltransferase activity (EC 2.4.2.10) and then converts OMP to uridine 5′-phosphate (UMP) via the OMP decarboxylase activity (EC 4.1.1.23). A computer modeling analysis of the experiments that led to the proposal [Traut, T. W., & Jones, M. E. (1977) *J. Biol. Chem. 252*, 8374–8381] that uridylate synthase channels intermediate OMP suggests that the experimental results do not demonstrate preferential use of OMP generated in the bifunctional complex as against exogenous OMP. This analysis shows that the experimentally observed amounts of [6-^{14}C]UMP from [6-^{14}C]orotate in the presence of various amounts of exogenous [7-^{14}C]OMP agree well with the amounts predicted by the computer simulations. Thus we conclude that uridylate synthase does not channel OMP. Additionally, the subsequent suggestion that channeling of OMP occurs to protect the intermediate from degradation by a nucleotidase [Traut, T. W. (1980) *Arch. Biochem. Biophys. 200*, 590–594] seems unlikely. The appropriate computer simulation demonstrates that low transient levels of OMP and protection of the intermediate are provided for strictly by the kinetic parameters of orotate phosphoribosyltransferase, OMP decarboxylase, and the nucleotidase. Additionally, calculations show that, in both sets of published experiments, the concentration of transient OMP greatly exceeded the concentration of OMP decarboxylase active sites. Thus, channeling of OMP by the bifunctional complex cannot be invoked to explain the evolution of uridylate synthase, and that event must be the result of some other selective pressure.

The last two steps in the de novo biosynthesis of pyrimidine nucleotides are carried out within a single polypeptide, uridylate synthase, in mammalian cells (McClard et al., 1980). The first three activities of this pathway are likewise found in another single protein species (Coleman et al., 1977), and indeed there is an increasing number of such multifunctional species appearing in the literature each year. Many possible explanations have been offered to explain the existence of such combined enzymatic activities; for reviews see Hammes (1981), Kirschner and Bisswanger (1976), and Bisswanger and Schminke-Ott (1980). Such complexes might exist for any, among others, of the following reasons: (1) simplification of the genetics of pathway expression, (2) the catalytic facilitation of a subsequent reaction of the generation of higher virtual concentration of substrate, (3) relief of pressure on the colligative solute capacity of ever more complex cells during the course of evolution (Atkinson, 1977), (4) simultaneous stabilization and/or facilitation of a concerted regulatory response to all enzymes involved in a pathway or subset of a pathway (Otsuki et al., 1982), and (5) protection of unique and reactive intermediates from unwanted side reactions or accumulation (Wasserman et al., 1983). Since OMP[1] is an intermediate unique to the de novo pyrimidine biosynthetic pathway, it might be tempting to suggest that the last explanation could provide the teleological rationale for the evolution of a bifunctional complex of orotate PRTase, which produces OMP, and OMP decarboxylase, which converts this intermediate to UMP.

Traut (1980) has pointed out recently that an extract from Ehrlich ascites carcinoma, which contains uridylate synthase, also contains a nucleotidase activity that has a measurable activity with OMP as substrate whereas yeast, which has no such activity, has separate decarboxylase and transferase activities. These observations led to the proposal that the mammalian enzyme thus evolved into a complex that could protect OMP from needless degradation by channeling the intermediate efficiently to UMP (Traut, 1980, 1982; Christopherson et al., 1981).

The conclusion from several years ago (Traut & Jones, 1977a) that UMP synthase channels the intermediate OMP (i.e., OMP formed on the bifunctional protein is preferentially decarboxylated over exogenous OMP) has been widely accepted in textbooks and reviews [see for example, Moyer and Henderson (1985)]. The experimental test of channeling was carried out according to



Orotate PRTase and OMP decarboxylase are represented by T and D, respectively. [7-^{14}C]OMP is an exogenous pool

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[1] Abbreviations: OMP, orotidine 5′-phosphate; UMP, uridine 5′-phosphate; OA, orotate; PRTase, phosphoribosyltransferase; PRPP, 5-phosphoryl-D-ribose 1-α-diphosphate; PP_i, diphosphate.

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in this case, and [6-^{14}C]OMP must be generated by the PRTase. It is expected that appropriate experiments using this system should reveal whether the amount of [6-^{14}C]UMP formed significantly exceeds the amount that would be predicted given no preferential use of [6-^{14}C]OMP, which would otherwise be free to equilibrate with exogenous [7-^{14}C]OMP. Our calculations, based on a computer model that numerically integrates the cumulative approximate differential rate laws of all independent enzyme-catalyzed processes, show that the amounts of [6-^{14}C]UMP synthesized are very close to the amounts predicted by following our computed time course of reaction. The previously predicted amounts (Traut & Jones, 1977a) are much smaller and, as we shall discuss in this paper, are not substantiated. We shall also discuss a similar problem with the paper by Pragobpol et al. (1984), that a particulate protozoan orotate PRTase/OMP decarboxylase channels OMP. Clearly, if uridylate synthase did not channel OMP within the bifunctional protein from Ehrlich ascites cells, then the suggestion (Traut, 1980, 1982) that the complex may have evolved for the purpose of protection of the intermediate would be moot. The low level of diversion of OMP to orotidine in vitro can readily be explained solely in terms of the kinetic properties of the nucleotidase and decarboxylase activities, and such degradation would not occur in vivo with or without channeling. This conclusion suggests that other explanations must be considered for the evolution of the uridylate synthase bifunctional protein. This work has been presented in preliminary form (McClard & Shokat, 1986).

Computational Methods

The criterion for channeling of an intermediate in a bifunctional enzyme is that the intermediate be consumed by the subsequent activity before it effectively dissociates into the bulk solvent (Duggleby et al., 1979). One experiment to test for this phenomenon is to challenge the intermediate with an isotopically labeled compound. In the case of uridylate synthase, which contains orotate PRTase and OMP decarboxylase activities, exogenously added OMP is the challenge against endogenous OMP produced by the transferase activity. Traut and Jones (1977a) employed different initial concentrations of exogenous OMP, [7-^{14}C]OMP, to determine the ability of uridylate synthase to channel [6-^{14}C]OMP. Our approach was to conduct a modeling experiment to determine whether the calculated amounts of [6-^{14}C]UMP predict the experimental result (no channeling) or fall significantly short of that amount (channeling verified). The progress of the challenge experiment was followed by generating a time course for the reaction, monitoring [7-^{14}C]OMP, [6-^{14}C]OMP (endogenously produced OMP), and [6-^{14}C]UMP (product from endogenously produced OMP, the channeling product). Equation 1 above represents the overall reaction sequence which the program models.

Once the kinetic parameters for each of the enzyme activities were selected, time courses for several different initial concentrations of exogenously added OMP were calculated by using an algorithm similar to that proposed by Storer and Cornish-Bowden (1974). Two types of competition experiments were performed by Traut and Jones (1977a). In the first, exogenous OMP was added at the same time [6-^{14}C]OA was added and the reaction was allowed to proceed for 20 min. In the second experiment, exogenous OMP was introduced into the reaction mixture 10 min after the reaction was initiated and the reaction was allowed to proceed an additional 20 min. Our model mimics that protocol and utilizes the standard Michaelis–Menten enzyme kinetic rate equation and confers no channeling advantage (i.e., the assumption of noninteracting sites) to the [6-^{14}C]OMP. The velocity calculation for the PRTase activity v_T is the standard rate expression with no modification since only [6-^{14}C]OA exists as a labeled substrate (see eq 1).

$$v_T = \left(\frac{V_{max}[[6\text{-}^{14}C]OA]}{K_{m(OA)} + [[6\text{-}^{14}C]OA]}\right)\left(\frac{[PRPP]}{K_{m(PRPP)} + [PRPP]}\right)$$

The velocity calculation for the decarboxylase activity is slightly more complex due to the fact that this activity needs to be partitioned into the velocities produced by endogenous and exogenous OMP. The expression depends only upon the relative concentration of each labeled compound with respect to the total OMP pool. Thus

$$v_{D6} = \left(\frac{V_{max}([[6\text{-}^{14}C]OMP] + [[7\text{-}^{14}C]OMP])}{K_{m(OMP)} + ([[6\text{-}^{14}C]OMP] + [[7\text{-}^{14}C]OMP])}\right) \times \left(\frac{[6\text{-}^{14}C]OMP}{([[6\text{-}^{14}C]OMP] + [[7\text{-}^{14}C]OMP])}\right)$$

where v_{D6} is the rate of OMP decarboxylase acting on [6-^{14}C]OMP; v_{D7} (for [7-^{14}C]OMP) is calculated similarly.

These rate expressions assume nonreversibility of each step. The PRTase-catalyzed step is essentially irreversible, owing to the fortuitous existence of a pyrophosphatase activity which cleaves the PP_i produced by the forward reaction (Traut & Jones, 1977a).[2] The decarboxylase step is essentially irreversible because CO_2 release in the forward reaction yields a highly favorable equilibrium constant as do the subsequent dephosphorylation reactions. Once each velocity was calculated it was multiplied by a very small time interval Δt to determine the amount of product formed during a given interval. The value of Δt was sequentially reduced until the concentrations of [6-^{14}C]OMP and [6-^{14}C]UMP at the end of the simulation had each failed to change in the fifth significant figure (using the same initial concentration of [7-^{14}C]OMP). Such a value of Δt was found to be 0.005 min. Smaller values of Δt were tested, but the added precision was not deemed worthwhile considering the increase in computation time.

The recursion program was used to calculate the rate of each reaction at time t_n with the substrate concentration at t_{n-1} and the kinetic parameters from Traut (1980) or values of V_{max} deduced from the experiments described by Traut and Jones (1977a). Once the rate at t_n for an enzyme was calculated, the small time interval Δt was multiplied by the velocity (v_n) to give the amount of product formed in the interval for the given enzymes. The amount of product formed in t_n, given by $\Delta t v_n$, was then added to the amount in the previous interval t_{n-1} to give the total in t_n. Thus for [6-^{14}C]UMP

$$[[6\text{-}^{14}C]UMP]_{t(n)} = [[6\text{-}^{14}C]UMP]_{t(n-1)} + \Delta t v_{D6(n)}$$

In the case of [6-^{14}C]OMP, two processes occur simultaneously—production of [6-^{14}C]OMP by the transferase and decarboxylation of [6-^{14}C]OMP to [6-^{14}C]UMP. Thus the incrementation becomes

$$[[6\text{-}^{14}C]OMP]_{t(n)} = [6\text{-}^{14}C]OMP_{t(n-1)} + \Delta t v_{T(n)} - \Delta t v_{D6(n)}$$

[2] Although OMP is a well-known inhibitor (competitive with respect to PRPP) of the transferase enzyme from lower organisms such as yeast (Victor et al., 1979), an inhibition term is not included in our simulation, owing to the observation (Traut & Jones, 1977a) that OMP actually stimulates the mammalian orotate PRTase to some extent.

The remaining incrementation equations are similarly determined:

$$[[7\text{-}^{14}C]OMP]_{t(n)} = [[7\text{-}^{14}C]OMP]_{t(n-1)} - \Delta t v_{D7(n)}$$

$$[[6\text{-}^{14}C]OA]_{t(n)} = [[6\text{-}^{14}C]OA]_{init} - \sum_{i=0}^{n} \Delta t v_{T(i)}$$

The modeling protocol is identical with the way the kinetic experiments were performed by Traut and Jones (1977a).

The system of coupled enzymes shown in eq 2 has also been used to investigate the ability of uridylate synthase to channel OMP (Traut, 1980). Orotate PRTase and OMP de-

$$\begin{array}{ccccccc} OA & \xrightarrow{T} & OMP & \xrightarrow{D} & UMP & \xrightarrow{P} & uridine \\ & & \downarrow P' & & & & \\ & & orotidine & & & & \end{array} \qquad (2)$$

carboxylase are denoted by T and D, respectively, and OMP and UMP are degraded by activities P′ and P, respectively. Although Traut (1980) regarded P′ and P as the same protein, they could be distinct (El Kouni & Cha, 1982). In the absence of a supply of ATP, the UMP produced is cleaved slowly to uridine (Traut, 1980). Since all of the kinetic parameters for each of the enzymes involved in the pathway are known, calculation of the time course for this overall process using computing techniques similar to those described above is possible. This model, similar to the first, treats the system depicted in eq 2 as if all of the enzymes were acting independently and obeying typical Michaelis–Menten kinetics. The rate equations for the transferase and decarboxylase are essentially as described above for the system depicted by eq 1. These rate expressions assume nonreversibility of each step according to the reasons stated above.

The system includes a competing reaction of OMP → orotidine which has been assumed to be catalyzed by a pyrimidine nucleotidase activity that also catalyzes the final reaction shown above, UMP → uridine (Traut, 1980). The ability of pyrimidine nucleotidase to catalyze OMP → orotidine is strictly dependent upon the number of active sites catalyzing UMP → uridine. The standard rate expressions for this case, in which a single enzyme acts upon two substrates that presumably compete for the same catalytic site (Pocklington & Jeffrey, 1969, and references cited therein), are

$$v_{P'} = \frac{V_{max}[OMP]}{[OMP] + K_m(1 + [UMP]/K_{m(UMP)})}$$

$$v_P = \frac{V_{max}[UMP]}{[UMP] + K_m(1 + [OMP]/K_{m(OMP)})}$$

By use of the above rate equations along with the published kinetic parameters (Traut, 1980), a computer program was employed to calculate the time course for the reaction progress. The recursion program, similar to that described for the system depicted by eq 1, calculates the rate of each reaction at time t_n with these kinetic parameters (Traut, 1980) and the substrate concentration at t_{n-1}. Progressively shorter increments of Δt were chosen until a value was obtained where the final values of the following parameters no longer changed in the fifth significant figure: (1) maximal concentration of transient OMP attained, (2) the time that the maximal OMP transient occurred, and (3) final concentration of orotidine produced.

Both programs were initially written in Applesoft BASIC and run on a 64K Apple II+ microcomputer, transcribed for a Macintosh computer with PASCAL, and later transcribed into a compiled version of PASCAL and run on a Digital VAX 11/780 mainframe for the smaller values of Δt. Copies of these programs are available from the authors on request.

Results

Traut and Jones (1977a) demonstrated that uridylate synthase can readily accept exogenous [7-^{14}C]OMP, which is then decarboxylated to UMP and $^{14}CO_2$ (see Figure 7 of that paper). It is clear that under the conditions of those experiments the average rate of maximal decarboxylation (V_{max} taking $[E]_t$ into account) was approximately 2.02 nmol min^{-1} by using the three points obtained with the highest (saturating, $\geq 25K_m$) concentrations of OMP; data obtained at lower concentrations of OMP are harder to interpret primarily because the [7-^{14}C]OMP challenge had, within experimental uncertainty and reasonable recovery of radioactivity, apparently been completely removed during the 20-min incubation. The value of V_{max} for orotate PRTase can be obtained by inspection of Figure 2 of the same paper. At 50 μM orotate (K_m = 1 μM) and 0.30 mM PRPP (K_m = 15 μM) the maximal rate was 0.77 nmol min^{-1} at 480 μg of protein, which was the amount used in the channeling studies (Traut & Jones, 1977a). If there were no competition from [6-^{14}C]OMP, it is clear that essentially all of the challenge ([7-^{14}C]OMP) would have been degraded before the 20-min incubation period ended for several initial concentrations of [7-^{14}C]OMP. As can be seen by the computer simulation shown in Figure 1A, the OMP challenge (10 μM) would be nearly completely gone in 12 min, with 8 min remaining without any challenge. Once the challenge had been exhausted, significant amounts of [6-^{14}C]UMP would then be formed from the [6-^{14}C]OMP that accumulated as a result on the initially high concentration of [7-^{14}C]OMP. Figure 1B illustrates a similar result based on our simulation of the published experiments in which the enzyme protein was preincubated with [6-^{14}C]orotate for 10 min followed by addition of [7-^{14}C]OMP and reaction for 20 min (Traut & Jones, 1977a). Only as the initial concentration of [7-^{14}C]OMP is raised to 50 μM or higher does the assumption that [6-^{14}C]-OMP is formed and [7-^{14}C]OMP is degraded linearly with time (Traut & Jones, 1977a) appear to hold (Figure 1C). Simulations like those shown in Figure 1 were carried out for all of the initial concentrations of [7-^{14}C]OMP used by Traut and Jones (1977a), and the calculated values of [6-^{14}C]UMP from our computer simulation are shown in Figure 2 along with the experimental and theoretical data from the experimental source. For the modeling of the 10-min delay experiment (Figure 2A), an additional curve was calculated by reducing both enzyme velocities by the same factor in order to exactly match the rate of [6-^{14}C]UMP synthesis in the experimental paper. The two simulations bracket the experimental results nicely and are far from the originally predicted values given by Traut and Jones (1977a). The experiment in which there was no delay in the addition of the challenge ([7-^{14}C]OMP) also yielded data in close agreement to our calculated values (Figure 2B). Small variations in K_m had little substantive effect on the simulations. Simulations performed on the data obtained at lower concentrations of enzyme protein and [6-^{14}C]OA [see Figure 5B of Traut and Jones (1977a)] gave similar results (data not shown).

Since the results of modeling studies described above do not support the likelihood of channeling of OMP in Ehrlich ascites cells, the hypothesis that uridylate synthase evolved in order to protect OMP from needless degradation by a nucleotidase that hydrolyzes the 5′-phosphate to form orotidine was reinvestigated. Traut (1980) calculated, by unspecified means, that, under the experimental conditions used to investigate the system depicted by eq 2, 8% of the [^{14}C]orotate would be lost to orotidine. Figure 3 shows the ratio of OMP decarboxylase

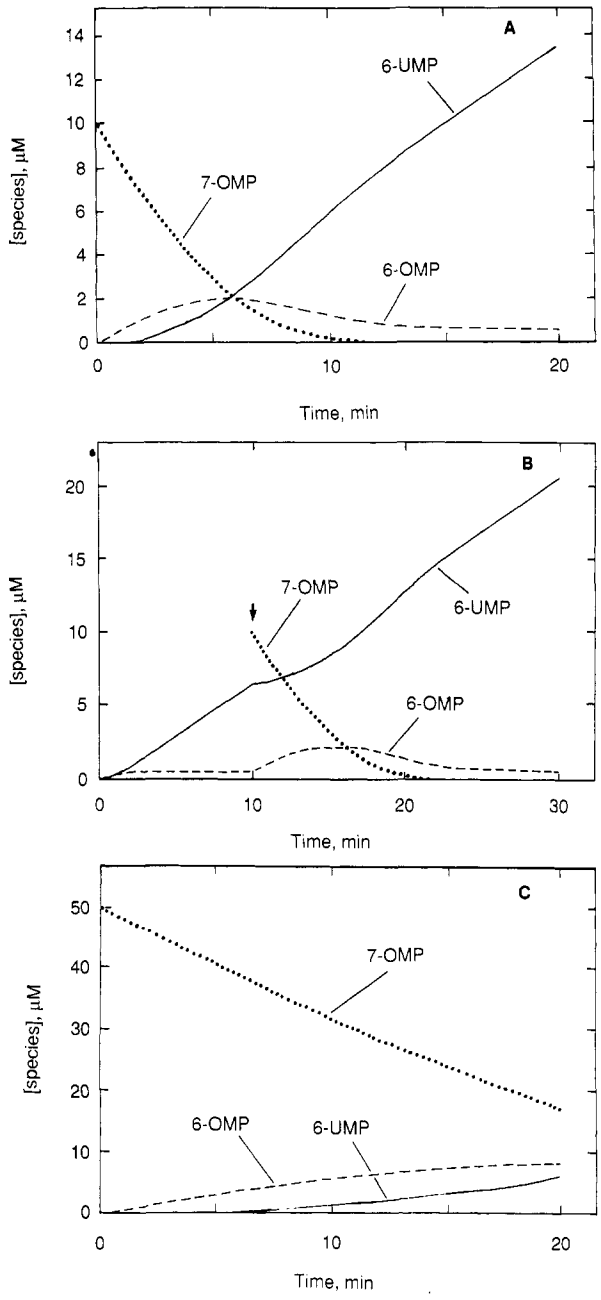

FIGURE 1: (A) Simulated time course corresponding to experiment of Traut and Jones (1977a), given by Figure 5A (open circles; 10 μM [7-^{14}C]OMP challenge) of that paper. v_D was 2.02 nmol min^{-1} and v_T was 0.77 nmol min^{-1}, as inferred from data in Figures 7 and 2, respectively, of their paper, as discussed in the text. The amount of [6-^{14}C]UMP predicted after 20 min is plotted in Figure 2B (pointed to by hollow arrow) of this paper. 7-OMP, [7-^{14}C]OMP; 6-OMP, [6-^{14}C]OMP; 6-UMP, [6-^{14}C]UMP. (B) Simulated time course corresponding to experiment of Traut and Jones (1977a), given by Figure 5A (closed circles; 10 μM [7-^{14}C]OMP challenge) of that paper. The OMP challenge was applied at time indicated by the arrow. The parameters v_D and v_T were as described above. The predicted amount of [6-^{14}C]UMP formed between 10 and 30 min (20 min after addition of [7-^{14}C]OMP, as calculated in the experimental paper) is plotted in Figure 2A (pointed to by hollow arrow) of this paper. (C) Simulated time course corresponding to experiment of Traut and Jones (1977a), given by Figure 5A (open circles; 50 μM [7-^{14}C]OMP challenge) of that paper. The parameters v_T and v_D were as described above. The predicted amount of [6-^{14}C]UMP after 20 min is plotted in Figure 2B (pointed to by solid arrow) of this paper.

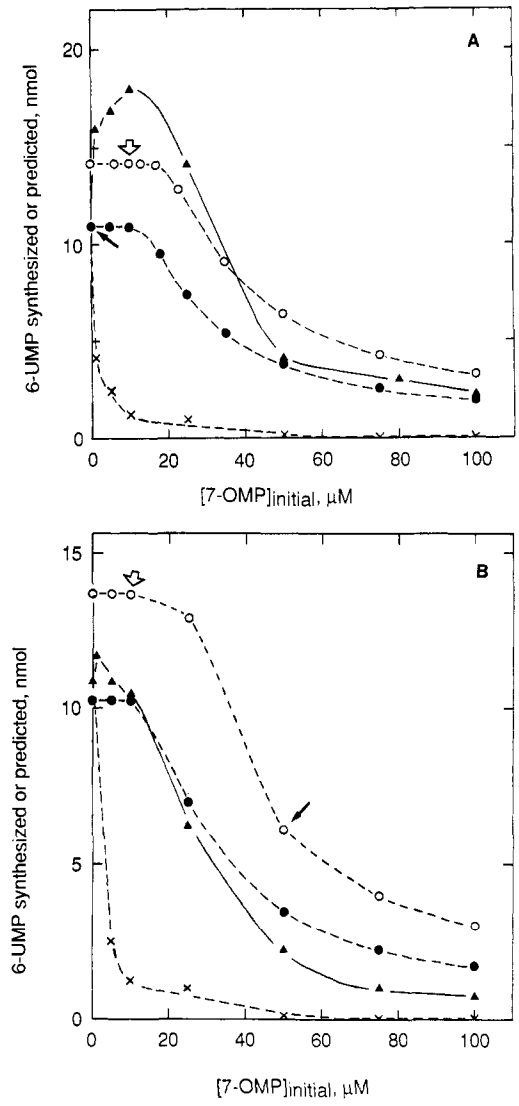

FIGURE 2: (A) Comparison of experimental data and predictions (Traut and Jones (1977a), Figure 5B, closed circles), shown as ▲ and × here, respectively, and amounts of [6-^{14}C]UMP predicted by computer simulation from reaction of [6-^{14}C]OA after addition of [7-^{14}C]OMP. Results of simulations shown by ○ were generated by using v_D = 2.02 nmol min^{-1} and v_T = 0.77 nmol min^{-1} as discussed in the text (point shown by hollow arrow was generated in Figure 1B). Data shown by ● were generated by reducing v_D and v_T to 1.55 and 0.59 nmol min^{-1}, respectively, in order to match the amount of [6-^{14}C]UMP observed experimentally (when [[7-^{14}C]OMP] = 0) by Traut and Jones (1977a); the points, indicated by the solid arrow, for ●, ▲, and × are thus all 10.9 nmol min^{-1} in the absence of exogenously added OMP. (B) Comparison of experimental data and predictions (Traut and Jones (1977a), Figure 5A, open circles), shown as ▲ and × here, respectively, and amounts of [6-^{14}C]UMP predicted by computer simulation from 20-min reactions of [6-^{14}C]OA in presence of various amounts of added [7-^{14}C]OMP. The values shown as ○ and ● were obtained from the same sets of parameters discussed above. The hollow arrow points to the result generated in Figure 1A, and the solid arrow points to the result generated in Figure 1C.

to nucleotidase activities that can be predicted from the concentration of OMP and the kinetic constants tabulated in the experimental paper. As [OMP] ⇒ 0, the ratio is limited by the ratio of the second-order rate constants (V_{max}/K_m), as expected. As the concentration increases, the nucleotidase activity becomes increasingly favored since it has an effectively higher V_{max}. The concentration of OMP reached experimentally does not exceed about 3 μM [see Traut (1980), Figure 1A], and the time-averaged value is less than that. Inspection of Figure 3 shows that no more than about 1% of the OMP produced can possibly be dephosphorylated to orotidine at any time. If one uses the more accepted value for K_m of 0.3 μM (Traut & Jones, 1977b; Jones et al., 1978; Jones, 1980a,b; Christopherson et al., 1981), the amount of diversion is about 0.3%. The value of 8% (Traut, 1980) is impossibly

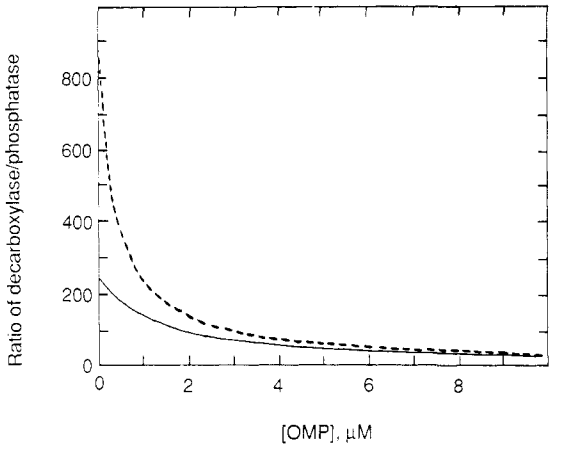

FIGURE 3: Ratio of decarboxylase to phosphatase activities acting on OMP. Calculations for the lower curve were performed by using kinetic parameters reported by Traut (1980). The upper curve was generated by using a K_m (OMP decarboxylase) of 0.3 μM (Jones et al., 1978; Christopherson et al., 1981; Jones, 1980a; Traut et al., 1980).

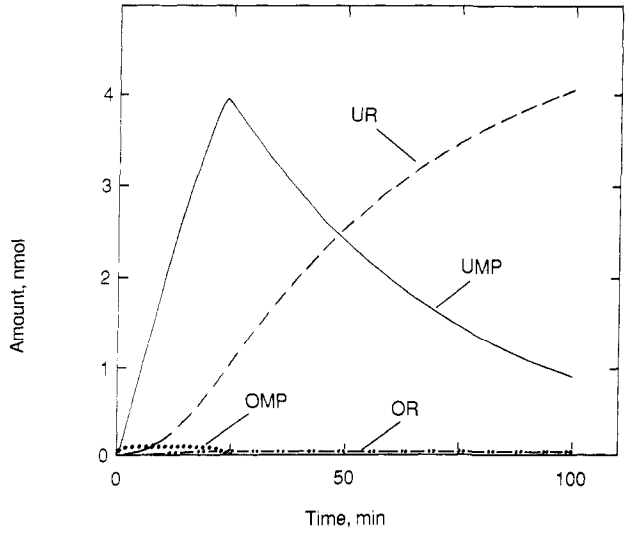

FIGURE 4: Simulation of system given by eq 2 for comparison with Figure 1A of Traut (1980). K_m values come from the work of Traut (1980), and V_{max} values are from Traut (1980) and divided by 3.9 to give the same crossover point of UMP and uridine. Species are labeled as follows: (—) UMP, (--) uridine (UR), (···) OMP, and (-··-) orotidine (OR).

high; that amount can only be obtained from a computation that does not account for competition by the decarboxylase. In view of the above considerations we expected to reproduce the experimental data using numerical integration of the combined derivative rate expressions for the system depicted in eq 2. As predicted, the simulated data in Figure 4 almost exactly mimic the experimental result [see Figure 1A of Traut (1980)]. The absolute velocities for the participant enzymes had little impact on the prediction of orotidine produced in our simulation.[3]

DISCUSSION

The results of our simulations demonstrate that the rates of synthesis of UMP from either OA or exogenous OMP by uridylate synthase can be explained without invoking a functional relationship (channeling) between the PRTase and decarboxylase sites as was previously believed (Traut & Jones, 1977a; 1978; Jones, 1980a,b; Traut, 1980, 1982; Traut & Payne, 1980; Christopherson et al., 1981). The large difference between amounts of [6-^{14}C]UMP predicted by our simulations and those predicted by Traut and Jones (1977a) apparently resulted from the assumption (Traut & Jones, 1977a) that those amounts could be estimated by averaging the concentrations of [7-^{14}C]OMP and [6-^{14}C]OMP at the beginnings and ends of the experiments. The assumption fails at low concentrations of [7-^{14}C]OMP, as can be inferred from the simulations in Figure 1A,B, but does appear to be correct at higher (e.g., $\geq$50 μM) concentrations (Figure 1C). The amounts of [6-^{14}C]UMP predicted by Traut and Jones (1977a) do not match those calculated from the analyses of our numerically integrated rate expression for the system depicted by eq 1. Our recalculations show that predictions made by using their assumption at higher concentration of OMP should be very similar to our simulated values.[4] Some of the experimental values exceed our computed values, but the small differences are certainly due to the slight stimulation of orotate PRTase caused by exogenous OMP and by preincubation for 10 min. Our modeling approach makes a minimum of assumptions and would be applicable to other similar problems.

In addition to the arguments presented above, there is one other consideration that casts doubt on the existence of channeling of OMP by uridylate synthase. It is expected that such channeling could only be operative if the number of active sites of the second enzyme either approximates or exceeds the number of molecules of the transient species, in this case [6-^{14}C]OMP. If one uses the OMP decarboxylase V_{max} (Traut & Jones, 1977a) as well as the turnover number and molecular weight per active site (McClard et al., 1980), one can calculate a maximum value[5] of 0.005 nmol of OMP decarboxylase sites in those assays. In the case of the addition of 10 μM [7-^{14}C]OMP, for example, 0.42 nmol of [6-^{14}C]OMP was found at the end of the 20-min incubation period (Traut & Jones, 1977a). The transient level is actually higher than that (approximately 2 μM, or 2 nmol), as demonstrated by the simulation shown in Figure 1B, and the "average" amount was about 1.2 nmol (1.2 μM). The ratio of those values reveals that the "channeled" OMP exceeds the concentration of active

[3] The report (Traut, 1980) that no orotidine was found experimentally may be explained by considering that 0.3–1% orotidine could have been undetected due to incomplete recovery of radioactivity; indeed the total moles of orotate are only accounted for to about 95% (see Figure 1; Traut, 1980), and under the conditions of the thin-layer chromatographic assay employed in that study, the typical loss of radioactivity (at the origin) was shown to be 3 ± 1% (Prabhakararao et al., 1975).

[4] Upon inspection of Figure 7 of the experimental paper (Traut & Jones, 1977a), one can calculate an average [[6-^{14}C]OMP] of 1.8 μM and average [[7-^{14}C]OMP] of 32.1 μM for the case where the initial [[7-^{14}C]OMP] was 50 μM. Assuming no channeling, one would predict that the decarboxylase would produce 2.1 nmol of [6-^{14}C]UMP from a calculation that employs the assumptions of Traut and Jones (1977a):

$$\text{nmol of [6-}^{14}\text{C]UMP} = \left(\frac{V_{max}t[\text{OMP}]_{total}(\text{av})}{[\text{OMP}]_{total}(\text{av}) + K_m}\right)\left(\frac{[[6\text{-}^{14}\text{C}]\text{OMP}](\text{av})}{[\text{OMP}]_{total}(\text{av})}\right) = \left(\frac{2.02 \text{ nmol min}^{-1} \times 20 \text{ min} \times 1.8\ \mu\text{M}}{33.9\ \mu\text{M} + 1.0\ \mu\text{M}}\right)$$

The authors calculated, by unspecified means, that essentially no (<0.1 nmol) [6-^{14}C]UMP would be generated. The value recalculated with their assumptions is much closer to our curve generated by the succession of time-course simulations. It is important to note that the authors observed the formation of 2.3 nmol of [6-^{14}C]UMP under the same conditions, except there was no preincubation that caused activation of the enzymes (see Figure 5A, open circles, of the experimental paper).

[5] The calculation of active sites uses the specific activity of OMP decarboxylase (McClard et al., 1980), which must be regarded as a minimal estimate. The number of active sites related to mass per unit of activity is a maximum estimate and thus the ratio of OMP to active sites is a minimum estimate.

sites by a factor of at least 200. Using established parameters of the pure protein (McClard et al., 1980) and the conditions of assay reported (Traut, 1980), one can calculate that, in the system depicted in eq 2, there was approximately 0.067 μM active sites. The experimental transient concentration of OMP was reported to be about 3 μM, which agrees with our value determined by simulation (Figure 4). The appropriate calculation shows that, during the steady-state phase of the reaction, the concentration of OMP exceeds the concentration of active sites by a factor of 60 and thus could protect less than 2% of the OMP produced by the transferase activity. Therefore, under the experimental conditions of assay reported in the experimental papers, channeling was not possible, yet under the conditions reported by Traut and Jones (1977a), about 30 times more synthesis of [6-^{14}C]UMP was observed than was predicted. Invoking an argument for channeling under those conditions requires that uridylate synthase be able to sequester greater than 200 OMP molecules [60 molecules or more from the report of Traut (1980)] at any one time and then to preferentially decarboxylate these "bound" OMP molecules.[6] The conclusion that a low transient time for OMP indicates the existence of channeling (Traut & Payne, 1980) can not be supported. Young et al. (1985), for example, have pointed out that transient time is extremely sensitive to the experimental conditions and that a lag may not be measured even without channeling. Indeed, such a condition is the goal in the design of a coupled enzyme assay, in which there is usually no functional relationship between the active sites of successive activities.

The hypothesis (Traut, 1980, 1982; Christopherson et al., 1981) that the presence of the OMP-degrading nucleotidase can be tied teleologically to a bifunctional complex that is capable of channeling is thus open to question from three angles. First, the theoretical evaluation of the amounts of [6-^{14}C]UMP formed from [6-^{14}C]OA (Figures 1 and 2) clearly reveals that these amounts can be explained by the kinetics of the independent enzymes. Second, as calculated above, the transient concentration of OMP greatly exceeds the concentration of active sites. Third, even if channeling were operative, OMP would only constitute a tiny portion of the myriad of possible nucleoside monophosphate molecules available as substrates; such competition would only augment the simplified situation illustrated by Figure 3, which considers only the competition by the decarboxylase. If one uses an estimate of 0.2 mM as the concentration of UMP in cells (Hitchings, 1973), assumes that the K_m and V_{max} values for each of the nucleotides are similar [indeed they are for UMP and OMP (Traut, 1980)], and uses the fact that the concentration of OMP in vivo is about 0.05 μM (Hitchings, 1973; Traut & Jones, 1977b), then only about 0.025% of the OMP could be lost with no competition from OMP decarboxylase even considered. When the latter is taken into account (refer to Figure 3), even with the upper estimate for K_m of OMP (1 μM) it is apparent that the fraction of OMP that would be lost to dephosphorylation under physiological conditions would only be 0.0001%. Thus, OMP levels are kept low by virtue of the kinetic parameters of the enzymes involved, and the evolutionary significance of uridylate synthase as a bifunctional protein cannot be for the purpose of protection of OMP from degradation. One cannot, therefore, reach conclusions about the associative properties of the PRTase and decarboxylase from other sources by using existence of an effective OMP phosphatase as a criterion (Rathod & Reyes, 1983). Since El Kouni and Cha (1982) have reported the probable existence of a unique OMP phosphatase, one must ask why such an activity would be necessary if OMP were being effectively completely sequestered.

Pragobpol et al. (1984) reported recently that orotate PRTase and OMP decarboxylase are associated with a particulate fraction from *Crithidia luciliae* that is capable of channeling endogenously produced [6-^{14}C]OMP with a preference factor of 50 over exogenously added [7-^{14}C]OMP. They applied the assumptions of Traut and Jones (1977a) in their computations, which were not detailed. The appropriate calculation[7] shows that there was probably a 400-fold excess of OMP molecules over active sites, and channeling would again be unlikely under such conditions. It is interesting to note that Pragobpol et al. reported that *Crithidia* does not possess an OMP-degrading activity, contrary to what would have been predicted (Traut, 1980; Rathod & Reyes, 1983).

It is reasonable to assume that sites catalyzing consecutive reactions must be overlapping or very proximal in order to channel an intermediate. Appling and Rabinowitz (1985) recently demonstrated that the dehydrogenase and cyclohydrase activities are indeed coordinately inhibited by antibodies that do not affect the synthetase site of the tetrahydrofolate trifunctional protein, which effectively channels 5,10-methenyl tetrahydrofolate between the dehydrogenase and cyclohydrolase (Wasserman et al., 1983). The apparent lack of channeling in the uridylate synthase bifunctional enzyme is in agreement with immunochemical studies that clearly demonstrated that the decarboxylase and transferase sites are very distinct (McClard & Jones, 1982). The observation that the transferase domain can become denatured with little or no effect on the decarboxylase site argues against the notion that the two sites are overlapping or even proximal. Indeed the active decarboxylase domain can be isolated after proteolytic removal of the transferase domain (Floyd & Jones, 1985). On the basis of the kinetic results of Traut and Jones (1977a), Traut (1982) and Floyd and Jones (1985) have pictured UMP synthase as a dimer of bifunctional peptides that are arranged in such a way that the decarboxylase domain of one subunit is proximal to the transferase domain of the other subunit and vice versa. Physical evidence of such an arrangement will be required before that model can be accepted.

There are numerous examples of multifunctional proteins that contain nonconsecutive activities [see, for example, Staples and Houston (1979), Keesey et al. (1979), and Huang et al. (1974)], and surely these protein species have evolved for purposes other than for intermediate channeling. Such proteins

[6] Although it is possible that as much as an additional 1 mol of OMP could bind per mole of active subunit at a putative regulatory site (Traut et al., 1980), a large excess of OMP over catalytic sites would remain.

[7] From the data in Figure 2 of Pragobpol et al. (1984), it is clear that their system accumulated a steady-state amount of 2.3×10^{-12} mol of OMP and that the rate of OMP decarboxylase was about 3.26×10^{-12} mol min^{-1}. Using their value of the molecular weight (70 000) and an assumed specific activity of 7.8 μmol min^{-1} mg^{-1} (McClard et al., 1980), one calculates there was approximately 8×10^{-15} mol of OMP decarboxylase sites in their channeling assays. It would be necessary to invoke a specific activity for pure *Crithidia* OMP decarboxylase of about 0.02 μmol min^{-1} mg^{-1} or lower to attain the degree of channeling reported; such a turnover number is unlikely since the value ranges from about 8 μmol min^{-1} mg^{-1} in mouse (McClard et al., 1980) to about 30–40 μmol min^{-1} mg^{-1} in yeast (Brody & Westheimer, 1979; Yoshimoto et al., 1978). It is worth noting that Pragobpol et al. (1984) measured small but finite channeling in their control experiments with the separate yeast enzymes. It is possible, of course, that a ^{12}C/^{14}C primary isotope effect as large as about 10% (O'Leary, 1978) could be manifested as apparent preference for [6-^{14}C]OMP (produced from OA "endogenously") if it were that ^{14}C substitution at C-7, but not at C-6, causes such an effect on the decarboxylation step.

that do catalyze sequential reactions may or may not channel their common intermediate. The chorismate mutase–prephenate dehydrogenase bifunctional enzyme from *Aerobacter aerogenes*, for example, catalyzes sequential reactions on the pathway to tyrosine biosynthesis, and the intermediate prephenate is completely sequestered because the two activities apparently share a single site (Heyde, 1979). In sharp contrast, the chorismate mutase–prephenate dehydratase bifunctional enzyme from *Escherichia coli* catalyzes sequential reactions on the pathway to phenylalanine, and the two active sites act completely independently (Duggleby et al., 1979). It is conceivable that as cells become increasingly more complex there may exist a greater need to simplify the genetic apparatus through a reduction in gene number or to reduce the number of particles that require limited solvent water (Atkinson, 1977). Accordingly, some reason other than channeling of OMP must be behind the evolution of the bifunctional uridylate synthase from separate gene products in lower organisms.

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Registry No. Uridylate synthase, 74870-74-9; orotidine 5′-phosphate, 2149-82-8; orotate phosphoribosyltransferase, 9030-25-5; orotidylate decarboxylase, 9024-62-8.

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