Substrate Interchanges and Oxygen Transfers Catalyzed by Glutamine Synthetase at Equilibrium*

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Experiments with isotopes are reported on the rates of various interchanges at equilibrium as catalyzed by glutamine synthetase from peas. A continued increase in the rate of the $P_i \leftrightarrow$ ATP interchange with increase in the glutamate and glutamine concentrations, and an increase in the rate of the $P_i \leftrightarrow$ ATP and glutamate $\leftrightarrow$ glutamine interchanges with increases in the concentrations of P_i and ATP, was observed. Such results eliminate the possibility of compulsory binding sequences with glutamate, glutamine, P_i, or ATP as the last reactant to bind. Effects of increase in NH_4^+ plus glutamine or of ADP plus ATP are consistent with partially compulsory pathways of substrate binding. The interchange of O^{18} between the γ-carboxyl group of glutamate and both the P_i and the terminal phosphoryl group of ATP was found to be considerably more rapid than was estimated from the extent of the $P_i \leftrightarrow$ ATP interchange. This demonstrates lack of spatial selectivity by the enzyme between at least two oxygens of bound P_i and adds further evidence to the previous conclusion that interconversion of bound substrates is a rapid step in the catalysis. Loss of O^{18} from glutamate as calculated for the extent of the glutamate $\leftrightarrow$ glutamine interchange gives evidence for a spatial selectivity by the enzyme between two oxygens of the bound glutamate.

Glutamine synthetase, which catalyzes the reaction

$$RC(=O)-O^- + ATP^{-4} + NH_4^+ \longleftrightarrow RCONH_2 + ADP^{-2} + HOPO_3^{-2}$$

will not catalyze a rapid isotopic interchange between any of its substrates unless all substrates are present (see Meister, 1962). Previous experiments (Boyer *et al.*, 1959) have shown that pronounced inequalities may occur in the equilibrium rate of interchange between orthophosphate (P_i) and ATP as compared to the rate of interchange between glutamate and glutamine. The principal purpose of this paper is to present further data on isotopic exchange rates catalyzed by glutamine synthetase under varied equilibrium conditions and to illustrate how such data can help give a better understanding of enzyme mechanisms. The data do not allow choice between reaction mechanisms involving a bound γ-glutamyl phosphate or anhydride of glutamic acid as an intermediate, as suggested by Krishnaswamy *et al.* (1960), or a concerted mechanism, as suggested by Buchanan and Hartman (1959), by Boyer (1960), and by Boyer and Graves (1962). The results do, however, give information pertinent to detection of slow steps and possible compulsory binding pathways in the catalysis, allow deductions about relative rates of various steps in the over-all catalysis, and give a novel approach to detection of possible spatial selectivity among oxygens of bound reactants.

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EXPERIMENTAL

Enzyme Preparation.—Glutamine synthetase was prepared from peas through stage 6 of a procedure described by Elliott (1953). Initial velocity measurements of the net reaction were made according to Boyer *et al.* (1959), except that the P_i formed was determined by measuring the absorbancy of the phosphomolybdate complex at 310 mμ (Berenblum and Chain, 1938). One unit of enzyme activity, defined for the following studies, is that amount of enzyme required to release 1 μmole of P_i per ml of reaction mixture per minute at 37°. No detectable ATPase activity was present in the enzyme preparation.

Neither of two preparations of glutamine synthetase tested was activated by preincubation with ATP in a manner shown by a previous preparation (Boyer *et al.*, 1959). The reason for this difference in behavior is not known.

Exchange Rate Measurements.—The rates of the $P_i \leftrightarrow$ ATP and $RCOO^- \leftrightarrow RCONH_2$ reactions at equilibrium were studied by adding trace amounts of uniformly labeled C^{14}-L-glutamate and P_i^{32} to equilibrium reaction mixtures described in the text. The rates of reactions giving interchange of oxygens among reactants were approximated by measurement of the disappearance or appearance of excess O^{18} originally added as glutamate or P_i. The amount of radioactivity added as P_i^{32}

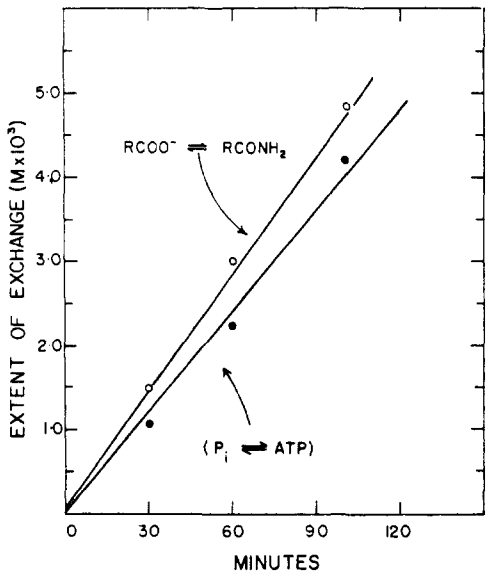

FIG. 1.—Time course of equilibrium exchange reactions. Reaction mixtures contained initially 20 mM glutamate, 20 mM glutamine, 40 mM P_i, 6 mM ATP, 0.1 mM NH_4^+, 10 mM mercaptoethanol, 20 mM $MgCl_2$, and approximately 0.4 units of glutamine synthetase in a total volume of 1 ml at *p*H 7.0 and 37°. After 5 minutes' incubation, trace amounts of P_i^{32} and C^{14}-glutamate were added to separate reaction mixtures. Aliquots were removed and analyzed for extent of interchange as discussed in the experimental section.

incorporated into ATP was determined as described by Boyer *et al.* (1959). Exchange of glutamate and glutamine was assessed first by separating the amino acids by paper chromatography in butanol–acetic acid–water, 4:1:5, and then by determining the amount of radioactive glutamine formed by scanning the chromatogram with an analytical count rate meter. Replicate determinations usually agreed within ± 4%.

Comparison of relative amounts of exchanges of different reactants does not necessitate that enzymic activity remain constant during the reaction period, although lack of enzyme inactivation is preferable. More important is assurance that isotope transfers observed do not result from net reaction. Appropriate choice of incubation conditions prior to isotope addition was made to assure that mixtures were sufficiently close to equilibrium so that negligible net reaction would be expected. In a number of experiments net reaction was ruled out by determination of reactant concentrations at the time of isotope addition and after additional incubation. In addition, in most experiments, presence of one or more reactants at very low concentration made net reaction negligible. Further assurance that equilibrium exchanges and not net reactions were being measured came from measurements on a typical reaction mixture at different time intervals, as given in Figure 1. The constancy of the equilibrium reaction rates with time showed both that enzyme activity was not decreasing and that net reaction was not responsible for the isotope redistribution.

For the data in Figure 1, as well as other data given herein, the total amount of interchange between any pair of reactants at a particular time was calculated as described previously (Boyer *et al.*, 1959), *i.e.*, from the following relation: amount of reaction $= \frac{-AB}{A+B} ln(1-F)$, where A and B are the molarities of the exchanging species and F is the fraction of isotopic equilibrium reached.

Preparation and Analysis of O^{18} Materials.—O^{18}-labeled glutamic acid was prepared according to Boyer *et al.* (1956), O^{18}-labeled phosphate was prepared as described by Cohn and Drysdale (1955). The amount of O^{18} in the γ-carboxyl group of glutamic acid was determined by heating a sample in an evacuated tube with a low flame, collecting the H_2O evolved from the conversion of glutamic acid to γ-pyrrolidone carboxylic acid by use of a dry ice–acetone trap, reacting the H_2O with guanidine HCl (Boyer *et al.*, 1961), and then determining the isotopic content of the resulting CO_2 by mass spectrometry. The O^{18} content of phosphate samples was determined by isolation as KH_2PO_4 and reaction with guanidine HCl (Boyer *et al.*, 1961). O^{18} in the phosphoryl groups of ATP was determined by hydrolysis in acid to yield P_i and proceeding as described above.

Phosphate, ADP, and ATP were precipitated as Ba salts from reaction mixtures deproteinized by $HClO_4$. P_i was separated from nucleotides by charcoal treatment (Crane and Lipmann, 1953). Glutamate was removed from the remaining reaction mixture by adsorption on a column of activated alumina (0.9 × 15 cm) in the HCO_3^- form (Darling, 1945). The adsorbed glutamate was eluted with 0.2 M NH_4HCO_3 and the NH_4HCO_3 was removed by prolonged sublimation under high vacuum.

Theoretical Equations for O^{18} Transfer from Glutamate.—When a large P_i-ATP pool is present, the transfers of O^{18} to and from the γ-carboxyl group of glutamate ($RCOO^-$) may be assumed to occur as follows:

(*a*) O^{18} loss occurs through transfer of one O from $RCOO^-$ to the P_i-ATP pool for every $RCOO^- \rightarrow RCONH_2$; and

(*b*) some O^{18} appearing in the $RCONH_2$ is returned to the $RCOO^-$ by the glutamate ⟷ glutamine interchange. O^{18} return from the P_i-ATP pool is negligible because of the large size of the pool and resultant small atom % excess. Equations describing O^{18} transfer for such a system may be derived as follows:

Let y = O^{18} content of glutamate at any time, z = O^{18} content of glutamine at any time, G = glutamate concentration, N = glutamine concentration, kG = amount of reaction per unit time, $\frac{kG}{G} = k$ = fraction of glutamate reaction per unit

TABLE I

COMPARISON OF THE LOSS OF O^{18} FROM GLUTAMATE TO THE GLUTAMATE-GLUTAMINE AND P_i-ATP EXCHANGES AND THE GAIN OF O^{18} IN P_i

Reaction mixtures contained initially 2 mM glutamate-O^{18} (1.95 atom % excess), 4 mM glutamine, 40 mM P_i, 6 mM ATP, 0.1 mM NH_4^+, 5 mM mercaptoethanol, 20 mM $MgCl_2$, and approximately 11 units of glutamine synthetase in a total volume of 20 ml at *p*H 7.0 and 37°. After 10 minutes' incubation, samples were removed for O^{18} analysis, traces of P_i^{32} and $RC^{14}OO^-$ were added, and the incubation was continued for a total of 70 minutes.

P^{32} in ATP at 70 min.	Calcd. $P_i \leftrightharpoons$ ATP in 10 to 70 min. Interval	C^{14} in $RCOO^-$ at 70 min.	Calcd. $RCOO^- \leftrightharpoons RCONH_2$ in 10 to 70 min. Interval	O^{18} in γ-COOH of Glutamate		
				10 min.	70 min.	Expected at 70 min.
%	*mM*	%	*mM*		*atom % excess*	
5.5	2.8	64	3.7	0.95	0.31	0.36[a]

[a] Calculated on the assumption that one oxygen was irreversibly lost to the large P_i + ATP pool for each $RCOO^- \leftrightharpoons RCONH_2$, using equations given in the experimental section.

time, $\frac{kG}{N} = k' =$ fraction of glutamine reacting per unit time,

$$\frac{dz}{dt} = k'y - k'z = k'(y - z)$$

$$\frac{-dy}{dt} = ky - \frac{kz}{2} = k\left(y - \frac{z}{2}\right)$$

The $-\frac{kz}{2}$ term arises because each glutamine → glutamate furnishes half of the oxygen of the glutamate γ-carboxyl, the other half coming from the large unlabeled phosphate pool.

One thus has two linear differential equations with two unknowns:

$$\frac{dy}{at} = k\left(\frac{z}{2} - y\right) \text{ and } \frac{dz}{dt} = k'(y - z)$$

Solution of these, with initial $y = 1$ and initial $z = 0$, gives

$$y = e^{\rho_2 t} + \frac{\rho_1 + k'}{\rho_1 - \rho_2}(e^{\rho_1 t} - e^{\rho_2 t})$$

$$z = \frac{k'}{\rho_1 - \rho_2}(e^{\rho_1 t} - e^{\rho_2 t})$$

where

$$\rho_1 = \frac{-(k + k') + \sqrt{(k + k')^2 - 2kk'}}{2}$$

$$\rho_2 = \frac{-(k + k') - \sqrt{(k + k')^2 - 2kk'}}{2}$$

For application to the glutamine synthetase experiment of Table I, let the original atom % excess O^{18} of glutamate = 1, $k = 1$, $G/N = {}^1/_2$; this means that the amount of the glutamate ↔ glutamine reaction would equal the molarity of glutamate in one arbitrary time unit. Also, since the molarity of glutamine is twice that of glutamate,

$$k' = \frac{k}{2} = \frac{1}{2}$$

From this, $\rho_1 = -0.191$ and $\rho_2 = -1.31$

therefore $y = e^{\rho_2 t} + \frac{e^{\rho_1 t} - e^{\rho_2 t}}{3.6}$

From the final equation, one may calculate various y values at different arbitrary times. For the data of Table I, at the time that radioactive isotopes were added, glutamate had 0.95 atom % excess O^{18} remaining from an original of 1.94 atom % excess. Thus the fraction of original isotope in glutamate = 0.95/1.94 = 0.49. From the estimations of y for different arbitrary time units, when 0.49 of original O^{18} is present as glutamate t would be close to 0.8. For the next 60 minutes the $RCOO^-$ $RCONH_2$ is equivalent to a t of 1.85 (total reaction ≈ 1.85 times the glutamate concentration) or a total equivalent arbitrary t of 2.65. At this t value, 0.185 of the original total O^{18}, equivalent to 0.36 atom % excess, would be expected to be present as glutamate if transfer of 0 were as assumed above.

RESULTS

pH Dependence of Interchange Rates.—Previous measurements have shown that considerable inequalities may exist in the rates of the $P_i \leftrightharpoons$ ATP and glutamate ↔ glutamine interchange reactions at equilibrium. The extent to which *p*H change may affect the relative rates had not been determined, and hence studies with one set of initial reactant concentrations were made. Results given in Figure 2 show that both exchanges occurred maximally at about *p*H 7.3. This is near the *p*H optimum for initial reaction velocity (Elliott, 1953). Such agreement may be fortuitious, since *p*H effects on equilibrium velocities are obviously complex.

Variation in Equilibrium Reactant Concentrations and Exchange Rates.—In multisubstrate enzyme systems, compulsory orders of substrate binding have important predictable effects on exchange rates at equilibrium (Boyer, 1959). For

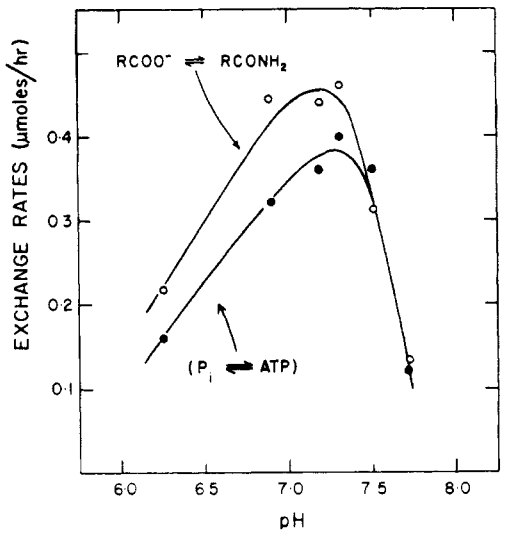

FIG. 2.—Dependence of exchange rates on *p*H. Conditions were as given with Figure 1 except the reaction mixture contained 1 mM glutamate and 2 mM glutamine.

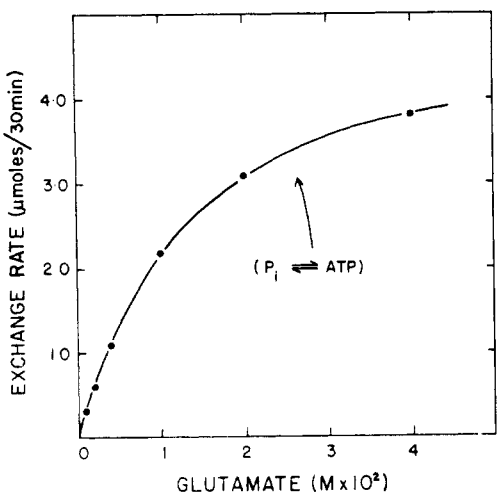

FIG. 3.—Effect of increase in glutamate and glutamine on the $P_i \leftrightarrow$ ATP exchange. Conditions were as given with Figure 1 except that the reaction mixture contained 8 mM ATP, 20 mM P_i, and concentrations of glutamate and glutamine indicated in the figure.

example, in the glutamine synthetase system, if glutamate or glutamine were the last reactant to add in a compulsory sequence, a continued increase in the concentration of glutamate and glutamine while other reactants remained constant and in equilibrium would be expected to give a continued increase in the glutamate $\leftrightarrow$ glutamine interchange rate but lead to a decrease in the $P_i \leftrightarrow$ ATP interchange rate. In contrast, if the reactants add randomly to the enzyme, a continued increase in glutamate and glutamine while equilibrium is maintained would lead to a continued increase in all interchange rates to a

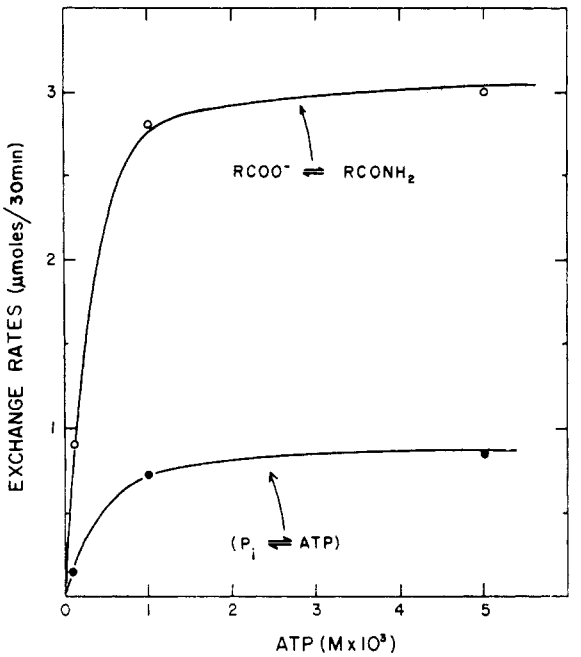

FIG. 4.—Effect of increase in P_i and ATP on exchange rates. Reaction mixtures contained initially 2 mM glutamate, 4 mM glutamine, and concentrations of P_i and ATP indicated in the figure. Other reactants and conditions were as described with Figure 1.

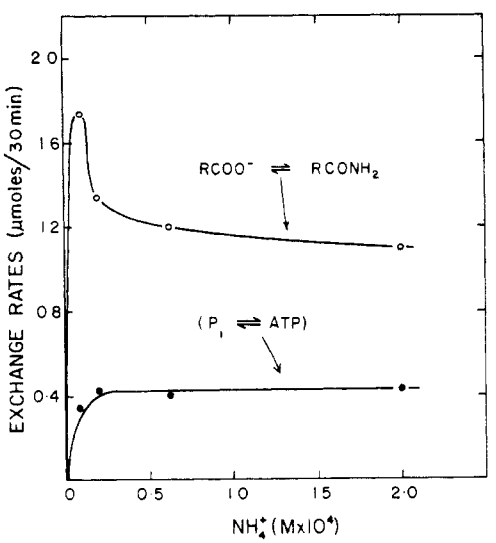

FIG. 5.—Effect of increase in NH_4^+ and glutamine on exchange rates. Conditions were as given with Figure 1 except that reaction mixtures contained initially 2 mM glutamate, 8 mM ATP, and concentrations of NH_4^+ and glutamine indicated in the figure.

maximum. The data in Figure 3 show the effects of an increase in the glutamate and glutamine concentrations on the $P_i \leftrightarrow$ ATP interchange rate. No decrease in the interchange rate was observed

even at a concentration of glutamate in a ten-fold excess of its K_m value (Boyer *et al.*, 1959).

The results of increasing the reactant pair, ATP and P_i, while maintaining equilibrium are shown in Figure 4. The highest final ATP concentration used was about 100 fold greater than its reported K_m (Boyer *et al.*, 1959). Such an increase resulted in increased rates of both the glutamate ⟷ glutamine and P_i ⟷ ATP interchange to an apparent plateau.

The effect of increasing amounts of NH_4^+ with a constant ratio of concentration of NH_4^+/glutamine is illustrated in Figure 5. Raising the NH_4^+ concentration to a value approximately five times its K_m value caused the P_i^{32} ⟷ ATP exchange to increase and then plateau, whereas in marked contrast the C^{14} glutamate ⟷ glutamine exchange first increased and then decreased. When the ADP concentration was elevated at a constant ADP/ATP ratio, a definitive decrease in the C^{14} glutamate ⟷ glutamine interchange occurred (Fig. 6). The P_i ⟷ ATP interchange was not as markedly affected, although some decrease was indicated.

Oxygen Transfer in Comparison to Substrate Interchange Rates.—In the net synthesis of glutamine by glutamine synthetase, one oxygen of the glutamate γ-carboxyl group appears in the P_i formed from ATP (Boyer *et al.*, 1956; Kowalsky *et al.*, 1956). Recognition that the covalent bond forming and breaking steps in the glutamine synthetase reaction are likely rapid compared to substrate release steps (see Boyer *et al.*, 1959) suggested the possibility that under equilibrium conditions oxygen transfer among reactants might exceed rates predicted from interchange of P^{32} or C^{14}. Several experiments making crucial comparisons were thus conducted.

In the experiment reported in Table I, oxygen loss from the γ-carboxyl group of glutamate was measured after establishment of equilibrium with a relatively large P_i plus ATP pool present. In experiments such as these, addition of a trace of O^{18}-containing compound after establishment of equilibrium is not technically feasible because of the relative insensitivity of the O^{18} analyses. Thus glutamate-O^{18} was present initially, and considerable O^{18} transfer occurred during the 10-minute incubation period prior to addition of radioactive glutamate and P_i. The O^{18} interchange rate was faster during the initial 10-minute incubation period than during the subsequent 60-minute incubation period. This likely reflects a decrease in the interchange rate as the ammonia concentration dropped to a low value during adjustment of the equilibrium. Determination of the O^{18} content of the glutamate at 10 and 60 minutes showed that the measured loss of O^{18} was close to that calculated on the assumption that one oxygen is lost from the γ-carboxyl group of glutamate for each glutamate ⟷ glutamine interchange.

In the experiment reported in Table II, a relatively large glutamate plus glutamine pool was present, and the rate of transfer of oxygen from the γ-carboxyl group of glutamate to the terminal phosphoryl group of ATP and to the P_i was measured. Comparison of the amount of oxygen transfer to the amount of P_i ⟷ ATP interchange showed two striking results, namely, (*a*) more oxygen was transferred to P_i than expected from the magnitude of the P_i ⟷ ATP interchange, and (*b*) more oxygen was transferred to the terminal phosphoryl group of ATP than expected from oxygen transfer to the P_i of the reaction medium and the magnitude of the P_i ⟷ ATP interchange.

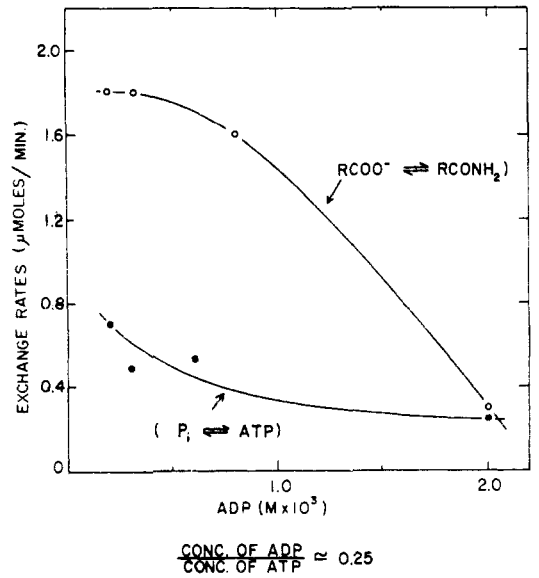

FIG. 6.—Effect of increase in ADP and ATP on exchange rates. Conditions were as given with Figure 1 except that reaction mixtures contained initially 2 mM glutamate, 4 mM glutamine, and concentrations of ADP and ATP indicated in the figure.

The experiment of Table III was performed as an alternate means of establishing oxygen transfers shown in the experiment of Table II. Conditions were similar to those used in Table II, except that O^{18} was initially present only in the P_i. If an active interchange of oxygens between the terminal phosphoryl group of ATP and the γ-carboxyl group of glutamate occurs, then less O^{18} from P_i should appear in the terminal phosphoryl group of ATP than calculated from the extent of P_i ⟷ ATP interchange. Similarly, O^{18} loss from P_i should be greater than calculated for loss of an oxygen for each P_i ⟷ ATP. The results show that both observed oxygen transfers far exceed the calculated values, thus corroborating the findings of Table II. The additional oxygen transfer from P_i to $RCOO^-$, above that estimated as in Table III, may be calculated to be equivalent to interchange between about 3.8 mM P_i and $RCOO^-$. Thus this interchange rate is about six times the P_i ⟷ ATP interchange rate.

In all experiments, interchange rates as measured at equilibrium were considerably slower than

TABLE II

TRANSFER OF O^{18} FROM GLUTAMATE TO P_i AND ATP AND OF P^{32} FROM P_i^{32} TO ATP

Reaction mixtures contained initially 20 mM glutamate-O^{18}, 20 mM glutamine, 2 mM P_i, 1 mM ATP, 0.02 mM ADP, 0.1 mM NH_4^+, 10 mM mercaptoethanol, 20 mM $MgCl_2$, and approximately 9 units of glutamine synthetase, in a total volume of 20 ml at *p*H 7.0 and 37°. The mixture was incubated for 5 minutes to attain equilibrium, then a trace of P_i^{32} added, samples were removed for O^{18} analysis, and the incubation was continued for a total of 50 minutes.

Compound or Group Analyzed	P^{32} Distribution at 50 Min.	Calcd. $P_i \leftrightarrow$ ATP During 5–50 min.	O^{18} Distribution		
			Observed		Expected at 50 min.
			5 min.	50 min.	
	%	*mM*	*atom % excess*		
γ-COOH of glutamate			1.33	1.31	
P_i	89		0.04	0.29	0.10[a]
Terminal phosphoryl of ATP	11	0.36	0.02	0.21	0.06[b]

[a] Calculated for transfer of one oxygen from glutamate to P_i for each $P_i \leftrightarrow$ ATP in addition to 0.04 atom % excess at 5 minutes, *i.e.* $1.33 \times \frac{0.36}{2} + 0.04 = 0.10$. [b] Calculated for transfer of three oxygens from P_i to ATP for each $P_i \leftrightarrow$ ATP, taking the O^{18} content of the phosphoryl group transferred as an average value of 0.16 atom % excess, *i.e.* $0.16 \times 0.36 = 0.06$.

TABLE III

TRANSFER OF O^{18} FROM P_i TO ATP AND OF P^{32} FROM P_i^{32} TO ATP

Conditions were as given with Table II, except initial concentration of ATP was 0.9 mM and O^{18} was present in P_i rather than glutamate. Final volume of reaction mixture, 30 ml. The mixture was incubated for 10 minutes to attain equilibrium, and then a trace of P_i^{32} added, samples were removed for O^{18} analysis, and the incubation was continued for a total of 100 minutes.

Compound or Group Analyzed	P^{32} Distribution at 100 min.	Calcd. $P_i \leftrightarrow$ ATP during 10–100 min.	O^{18} Distribution		
			Observed		Expected at 100 min.
			10 min.	100 min.	
	%	*mM*	*atom % excess*		
P_i	76		1.06	0.69	0.94[a]
Terminal phosphoryl of ATP	24	0.62	0.06	0.18	0.60[b]

[a] Calculated as follows: Increase in O^{18} of the terminal phosphoryl of ATP in the 10–100 minute interval, 0.13 atom % excess, is equivalent to loss of $0.13 \times \frac{3}{4} \times \frac{0.9}{2.0} = 0.04$ atom % excess O^{18} from P_i. Additional loss expected from irreversible transfer of one oxygen from P_i to $RCOO^-$ for each $P_i \leftrightarrow$ ATP $= 1.06 \times \frac{0.62}{2.0} \times \frac{1}{4}$ $= 0.08$ atom % excess. This gives an expected value of $1.06 - 0.12 = 0.94$ atom % excess. [b] Calculated for transfer of three oxygens from P_i to ATP for each $P_i \leftrightarrow$ ATP, taking the O^{18} content of phosphoryl group transferred as an average value of 0.88 atom % excess, *i.e.* $0.88 \times \frac{0.62}{0.90} = 0.60$.

the expected rate of initial net reaction under optimal conditions with the same amount of enzyme. For example, the amount of enzyme used for the experiments of Table I could catalyze a net formation of about 33 mM glutamine during the 60-minute incubation period if optimal initial conditions could be maintained. Only a 3.7 mM glutamate ↔ glutamine interchange was observed, however. For the experiments of Table II, the amount of enzyme used could catalyze net formation of about 20 mM glutamine during the 45-minute incubation but only a 0.36 mM $P_i \leftrightarrow$ ATP interchange was observed. Such results are expected because of the presence of a variety of combinations of enzyme with substrates under equilibrium conditions. Expressions for equilibrium rates contain both reactant and product terms in the denominator in contrast to only reactants in initial velocity rate expressions.

DISCUSSION

Implications of these findings for the reaction mechanism can be visualized with the aid of a simple illustration giving the dissociation and interconversion reactions for glutamine synthetase (Fig. 7). This diagram depicts the binding of ammonia, glutamate, and ATP in steps 1, 2, and 3, the interconversion of the bound substrates within the enzyme "box" (step 7), and the release of glutamine, P_i, and ADP in steps 4, 5, and 6. Deductions from the oxygen transfer data are

based on the probability that the only way glutamine synthetase can catalyze transfer of an oxygen of the γ-carboxyl group of glutamate to P_i is through cleavage of ATP to ADP and P_i with concomitant formation of a covalent glutamyl derivative. The most probable covalent glutamyl derivative to be formed is, of course, glutamine, but present data do not rule out the possibility of formation of a glutamyl-enzyme which is cleaved by ammonia. Incorporation of oxygen from P_i into the γ-carboxyl of glutamate would involve the reversal of the above reactions. Oxygen transfers between substrates of the reaction medium are thus considered to involve as a minimum the interconversion of bound substrates as depicted by step 7 in Figure 7, and interchange of bound and free forms of the substrates between which oxygen transfer is observed.

The observed inequalities of the $P_i \leftrightarrows$ ATP and glutamate $\leftrightarrows$ glutamine exchange rates means, as indicated earlier (Boyer *et al.*, 1959), that the interconversion of the bound substrates, as depicted by step 7 within the box, is more rapid than rate-limiting substrate release and binding steps. The occurrence of more rapid oxygen transfer than predicted from the rate of $P_i \leftrightarrows$ ATP interchange adds weight to this conclusion. If the interconversion of bound substrates were markedly slower than the interchanges between free and bound substrates, than all interchange rates between substrates and products would be equal, and oxygen transfers would occur in accordance with over-all substrate interchange rates.

The experiments illustrated in Figures 3 and 4 suggest that ATP and glutamate as well as P_i and glutamine bind independently to the enzyme. A compulsory pathway of reactant addition would have demanded a decrease in either the $P_i \leftrightarrows$ ATP or glutamate $\leftrightarrows$ glutamine interchange. The fact that the K_m for glutamate remains approximately constant at low and high levels of ATP also suggests a random order of reactant addition (Boyer *et al.*, 1959). Data of Krishnaswamy *et al.* (1960) with highly purified glutamine synthetase from sheep brain indicated that C^{14} added as glutamate sediments with the enzyme only in the presence of ATP. The authors suggested a compulsory pathway in which ATP was bound first by the enzyme, and this may reflect a fundamental difference between the sheep and pea enzymes. Their data are, however, also consistent with a random order of addition of ATP and glutamate. Sedimentation of C^{14} added as glutamate with the enzyme would be expected independent of whether or not a compulsory pathway existed if an undissociable intermediate was formed from the reaction of ATP and glutamate.

The experiments with increasing ammonia and glutamine show that an increase in the NH_4^+ concentration decreases the glutamate $\leftrightarrows$ glutamine interchange (Fig. 5) to a lower level but apparently not to zero. This is consistent with

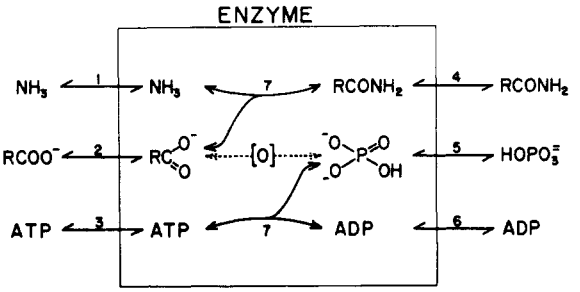

FIG. 7.—A schematic representation of the glutamine synthetase reaction at equilibrium.

the presence of bound ammonia decreasing but not preventing the disssociation of glutamate. Previous kinetic studies, showing lack of any effect of NH_4^+ concentration on the K_m for glutamate (Boyer *et al.*, 1959), give no evidence for NH_4^+ favoring glutamate binding. A compulsory pathway could exist under steady state conditions however, and be obscured by a fortuitous equality among rate constants.[1] It must be emphasized, however, that a decrease in interchange of glutamate $\leftrightarrows$ glutamine is suggestive of a partially compulsory pathway but does not constitute proof.

The decrease in the glutamate $\leftrightarrows$ glutamine interchange with increase in the ATP and ADP (Fig. 6) is consistent with a mechanism in which the dissociation of glutamine is inhibited by enzyme-bound ADP. The marked decrease in the glutamate $\leftrightarrows$ glutamine exchange and slight decrease in the $P_i \leftrightarrows$ ATP exchange could also result, however, if the ADP in some manner forms inactive complexes with the enzyme. This might

[1] For example, for an enzymic reaction with a compulsory pathway,

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAB$$

$$EAB \xrightarrow{k_3} E + \text{products}$$

The initial velocity expressions under steady-state conditions (Segal *et al.*, 1952) lead to the following: Apparent K_m for

$$A = \frac{k_{-1}/k_1[k_3/k_{-1} + (k_2 + k_3)/k_2(B)]}{1 + (k_2 + k_3)/k_2(B)}$$

and apparent K_m for

$$B = \frac{(k_2 + k_3)[1 + k_{-1}/k_1(A)]}{1 + k_3k_1(A)}$$

From this it follows that if $k_{-1} = k_3$, *i.e.*, the tendency of EA to give free E equals that of EAB to give free E, variation of concentration of one substrate will have no effect on the apparent K_m of the other substrate.

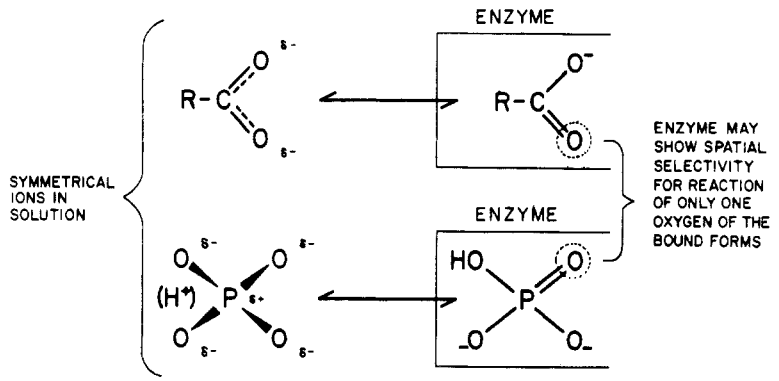

FIG. 8.—Schematic representation of possible spatial selectivity among oxygens of bound carboxylate and orthophosphate.

result from ADP binding at the glutamate or the P_i site, for example. An increase in the concentration of ADP and ATP should not decrease the rate of ADP $\leftrightarrows$ ATP interchange if ADP is the last reactant in a compulsory sequence. Experiments to test this possibility have not as yet been performed.

The data on rate of oxygen transfer between substrates are pertinent not only to relative rates of certain reaction steps but also to possible spatial selectivity among oxygen atoms of bound reactants. Such possible selectivity is indicated by consideration of the spatial arrangement of free and enzyme-bound reactants as depicted in Figure 8. The carboxylate and phosphate ions in solution are symmetrical, but, upon binding to the enzyme, electronic charges may become localized and the position of oxygens relative to the enzyme surface and other bound reactants may become fixed. Thus only one oxygen of the bound glutamate might be available for cleavage of ATP, and only one oxygen of bound orthophosphate might be available for cleavage of bound glutamine in the reverse reaction.

The data of Tables II and III demonstrate that the interchange of oxygen between the γ-carboxyl group of glutamate and the terminal phosphoryl group of ATP can occur without passage of the oxygen through P_i of the medium, and, further, that interchange of oxygen between the γ-carboxyl of glutamate and P_i can occur without interchange of P_i and ATP. Such results demonstrate that there is a lack of spatial selectivity between at least two oxygens of bound P_i and that the substrate interconversion step is rapid compared to the binding and release of P_i and ATP. In terms of the reaction scheme depicted in Figure 7, the oxygen transfer may occur by the following steps: (*a*) Interchange between free glutamate-O^{18} and bound glutamate, (*b*) interconversion of bound substrates to give bound P_i-O^{18}, (*c*) reversal of the interconversion step before release of bound P_i and with transfer of O^{18} to the terminal phosphoryl group of ATP; this would not occur if there were absolute spatial selectivity for the oxygens of bound P_i because the same oxygen received from glutamate would be returned to glutamate, and (*d*) interchange between free and bound ATP. A lack of spatial selectivity among P_i oxygens without release of bound P_i could result if P_i were bound momentarily through only one oxygen with resultant temporary rotational freedom.

The data of Table I, taken together with other equilibrium rate data, give strong indication but not definitive proof of a spatial selectivity between the oxygens of bound glutamate. The results show that the loss of O^{18} from the γ-carboxyl of glutamate was as calculated for the loss of only one oxygen for each interchange between the glutamate and the glutamine of the medium. If either oxygen of the γ-carboxyl group of bound glutamate had an equal possibility for reaction, the occurrence of a rapid interconversion step (step 7 of Fig. 7), together with lack of spatial selectivity between oxygens of bound P_i, would be expected to result in interchange of both oxygens of bound glutamate with bound P_i. Under such conditions, loss of O^{18} from the glutamate would be more rapid than expected from the over-all glutamate $\leftrightarrows$ glutamine interchange rate.

Other deductions about relative rates of reaction steps are also possible from the data of Table I. Interchange through steps 2, 7, and 4 of Figure 7 must occur at a faster rate than interchange through steps 2, 7, and 5, otherwise O^{18} from the γ-carboxyl of glutamate could be transferred to P_i without glutamate $\leftrightarrows$ glutamine interchange. Thus interchange between free and bound P_i must be less than between free and bound glutamine under the conditions of this experiment. Similarly, interchange through steps 2, 7, and 4 must occur more rapidly than interchange through steps 2, 7, and 3 under the conditions of this experiment; otherwise O^{18} transfer to the terminal phosphoryl group of ATP would give an O^{18} depletion from glutamate exceeding that calculated from the glutamine $\leftrightarrows$ glutamate interchange rate.

Exchange patterns such as those found for

glutamine synthetase may be rather general for other similar enzymes. Inequalities of exchange rates in the acetate thiokinase reaction have been reported (Boyer *et al.* 1959). Studies by Hager (1957) with succinate thiokinase at equilibrium showed a more rapid interchange of the oxygens of succinate with P_i than interchange of P_i and ATP. Hager's deductions were quite limited, however, and differ from the interpretation given here. Our interpretation would be that the observed oxygen transfer could result from a relatively slow interchange between free and bound ATP compared to the interchange between free and bound succinate, the interconversion of bound succinate to bound succinyl-CoA with formation of bound P_i, and interchange between free and bound P_i.

A spatial selectivity between the oxygens of the γ-carboxyl of bound glutamate implies a marked difference in reactivity of bound compared to free glutamate and adds plausibility to the suggestion that bound glutamate and ammonia might react concurrently with ATP cleavage in a concerted reaction (Buchanan and Hartman, 1959; Boyer, 1960) or actually prior to ATP cleavage (Boyer *et al.*, 1959).

The various deductions given in this paper, together with other information, add weight to a concept of substrate activation accompanying binding with little appreciable shift in the position of bound reactants during enzymic catalysis. The rapid interconversions of bound substrates can be visualized as occurring by a concerted reaction through one single key transition state. Such a mechanism has been termed a "key transition state" hypothesis for enzyme catalysis (Boyer and Graves, 1961). It must be emphasized, however, that present information does not eliminate the possible formation of a tightly bound γ-glutamyl phosphate as a discrete catalytic intermediate (see Krishnaswamy *et al.*, 1960, and Meister, 1962).

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