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Dynamic Reversal of Enzyme Carboxyl Group Phosphorylation as the Basis of the Oxygen Exchange Catalyzed by Sarcoplasmic Reticulum Adenosine Triphosphatase†

Paul D. Boyer,* Leopoldo de Meis, Maria da Gloria Costa Carvalho, David D. Hackney

ABSTRACT: Millisecond mixing and quenching experiments demonstrate an apparent $t_{1/2}$ for the labeling of phosphorylated sarcoplasmic reticulum ATPase by $^{32}P_i$ at pH 6 and 30 °C of 30 to 40 ms. Under the same conditions, the rate of exchange of water oxygens with inorganic phosphate (P_i) is about 40 mol of H_2O exchanged with P_i per 10^6 g of protein per s. Theoretical equations are developed for the expected ^{32}P-labeling pattern given various comparative rates of flux between P_i and the Michaelis complex and between the Michaelis complex and phosphorylated enzyme. The results show that the rapid reversal of the formation of the phosphorylated enzyme is a major source of the oxygen exchange and are consistent with such reversal being the only source.

The sarcoplasmic reticulum ATPase of skeletal muscle has been shown to catalyze a rapid exchange between oxygens of inorganic phosphate and water (a P_i[1] $\rightleftharpoons$ HOH exchange) in the presence of Mg^{2+} (Kanazawa and Boyer, 1973). The cleavage of ATP by the enzyme proceeds with intermediate formation of a phosphoenzyme (see Hasselbach, 1974) and a phosphoenzyme can be formed from P_i in the presence of Mg^{2+} and absence of added adenine nucleotides or a Ca^{2+} gradient (Kanazawa and Boyer, 1973; Masuda and de Meis, 1973; Kanazawa, 1975; de Meis, 1976). The phosphoryl group has been demonstrated to be attached to the carboxyl group of the side chain of an aspartic acid residue (Degani and Boyer, 1973) and is formed by displacement of a water oxygen from P_i (Dahms et al., 1973). These findings suggested that the rapid $P_i \rightleftharpoons$ HOH exchange may result from dynamic reversal of the formation of the phosphoenzyme from enzyme and P_i.

The purpose of the studies reported herein was to use rapid mixing and quenching techniques to measure the rate of formation and cleavage of the phosphoenzyme to find if this reaction may account for the oxygen exchange. Such measurements contribute to the understanding of the catalysis by the ATPase and are relevant to the mechanism of other enzyme-catalyzed oxygen exchanges.

Experimental Procedures

Phosphoenzyme Formation and Measurement. The phosphorylation of the sarcoplasmic reticulum ATPase was conducted with membrane vesicle preparations from rabbit skeletal muscle under conditions and with materials as described elsewhere (Masuda and de Meis, 1973; de Meis, 1976). The experiments were designed to measure the rate of interchange between P_i and the phosphoenzyme (E-P) under steady-state conditions. This has the advantage that any possible changes in rates of reaction steps accompanying the initial exposure of the enzyme to P_i are avoided. Thus the enzyme preparation was incubated with unlabeled P_i for about 10 min before filling of the syringes for the rapid mixing. The reaction mixture containing enzyme was then mixed rapidly with an equal volume of a solution of identical composition but without enzyme and with $^{32}P_i$ (approximately 10^8 cpm per ml) present, followed by quenching upon mixing with an approximately equal volume of 0.89 M perchloric acid. Control samples for measurement of total E-P were incubated with $^{32}P_i$ for 20 s, a period quite sufficient for maximal formation of phosphoenzyme under the reaction conditions. The amount of ^{32}P-labeled phosphoenzyme in the quenched samples was measured as previously described (Masuda and de Meis, 1973).

The P_i concentration used (6 mM) was approximately three times the concentration required for half-maximal E-P for-

† From the Department of Chemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024 (P.D.B. and D.D.H.), and Institute de Biofiscia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, GB, Brazil (L.deM. and M.deG.C.C.). *Received June 25, 1976* (supported in part by National Science Foundation Grant PCM 75-18884 and by the Consello Nacional de Deservolvimento Cientifics e Tecnologies, Brasil. D.H. was supported by a Postdoctoral Fellowship from the Institute of General Medical Sciences, United States Public Health Science. M.deG.C.C. was supported by a fellowship from the Consello Nacional de Deservolvimentio Centifico e Tecnologies, Brasil.

[1] Abbreviations used: P_i, inorganic phosphate; E-P, phosphoenzyme.

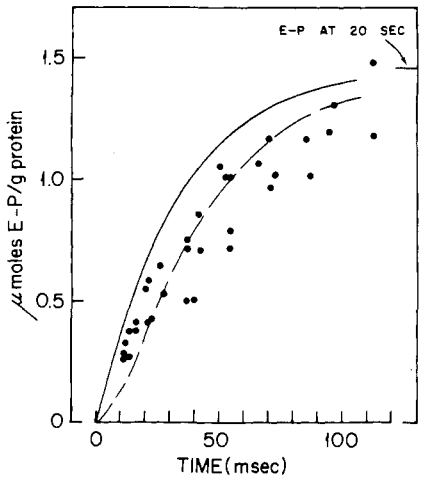

FIGURE 1: Rate of labeling of phosphoenzyme by $^{32}P_i$. A 1-ml volume of reaction mixture in one syringe contained 0.67 mg of protein as sarcoplasmic reticulum vesicles in 20 mM Tris-maleate buffer, 30 mM $MgCl_2$, 15 mM EGTA, and 6 mM P_i at pH 6.0 and 30 °C. The suspension was mixed with an equal volume of reaction medium without ATPase but with $^{32}P_i$ present (see Experimental Procedures) and quenched at the indicated times with 2 ml of 0.89 M $HClO_4$ containing 10 mM P_i. The solid and dashed lines are theoretical curves calculated as described in the text.

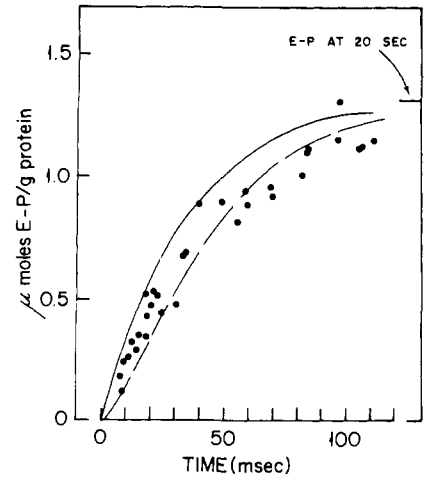

FIGURE 2: Rate of labeling of phosphoenzyme by $^{32}P_i$. Conditions were as given in Figure 1 except 1.3 mg of protein was present. The solid and dashed lines are theoretical curves calculated as described in the text.

mation (Masuda and de Meis, 1973), assuring near-maximal enzyme phosphorylation at longer time periods in our experiments.

Rapid Mixing and Quenching. The mixing–quenching experiments were performed with a simple three-syringe mixer described elsewhere (Smith et al., 1976). Mixing of the solution equilibrated with the enzyme and the reaction mix containing $^{32}P_i$ occurs in the first Y-tube mixer; quenching with perchloric acid in the second Y tube.

Oxygen Exchange Rate Measurements. At the same time the rapid mixing experiments were conducted, reaction mixtures of identical composition but with $H^{18}OH$ present were incubated for periods of 3 to 30 min and then quenched with perchloric acid. P_i was isolated from the quenched solution and its ^{18}O content measured essentially as described elsewhere (Boyer and Bryan, 1967).

Results

The Rate of Phosphoenzyme Labeling. Preliminary experiments demonstrated that the rate of labeling was indeed in the range anticipated for explanation of the oxygen-exchange measurements, but that some variability was encountered in the measurements of the amount of [^{32}P]E–P present. Experiments were thus designed so that a relatively large number of samples could be obtained under conditions appropriate for covering the range between little and near-maximal E–P formation within the limitations of the rapid mixing–quenching device. Results of two experimental series of measurements covering reaction times from 0 to slightly greater than 100 ms and with two different levels of enzyme present are given in Figures 1 and 2. Also shown in the figures are the amounts of labeled E–P found in separate 20-s incubations. The results show that, with little or no lag, a rapid labeling of the E–P present occurs, approaching maximal labeling within 100 ms reaction time. Apparent $t_{1/2}$ for labeling is 30–40 ms. The total amount of E–P present in these experiments (1.31 and 1.44 μmol per mg of protein for experiments of Figures 1 and 2, respectively) is similar to that noted earlier (Masuda and de Meis, 1973; de Meis, 1976) and, on the basis of approximation of the amount of ATPase present, represents phosphorylation of about 20–25% of the probable amount of active site present (MacLennan and Holland, 1975).

TABLE I: Observed Rates of Oxygen Exchange.[a]

Experimental Series	Time of Incubation (min)	Obsd ^{18}O in P_i (atom % excess)	Atom Fraction Exchange	Exchange Rate (μM/s)[b]
See Figure 1	10	0.094	0.49	27
	30	0.161	0.84	25
	30	0.166	0.87	27
See Figure 2	3	0.050	0.26	42
	3	0.060	0.31	47
	10	0.140	0.73	54
	10	0.142	0.74	55

[a] Samples were incubated under conditions identical with those as described for Figures 1 and 2, except that 0.506 atom % excess of ^{18}O was present in the water. The atom fraction exchange was calculated from the observed atom % excess of ^{18}O in the P_i with appropriate corrections for carrier P_i added with the perchloric acid quench. The total μatoms of water oxygen exchanged with P_i was calculated from the fraction exchange as described previously (Dempsey et al., 1963). [b] Exchange rate is expressed as micromolarity of water oxygens exchanging with P_i per second.

The lines on the figures represent theoretical curves for the labeling expected for selected rates of reaction steps calculated as given later in the Discussion.

Approximately twice as much enzyme was used in experiments for Figure 2 as for Figure 1. As anticipated, the rate of labeling of E–P appears to be independent of the enzyme concentration.

The Rate of Oxygen Exchange. The observations for measurement rates of oxygen exchange for samples incubated under conditions as used for the experiments of Figures 1 and 2 are summarized in Table I. For the observations made under conditions as used for Figure 2, the rates from the 10-min incubation were used for later calculations as the lower amount of oxygen exchange in the 3-min samples made those results less accurate. Close to the same rate of exchange was observed with both samples when calculated on the basis of amount of enzyme present. The rate found in these experiments (pH 6, 30 °C, 6 mM P_i) of about 40 mol of H_2O exchanged with P_i per 10^6 g of protein per s may be compared with that rate of

about 500 mol reported by Kanazawa and Boyer (1973) at pH 7, 37 °C, 40 mM P_i. Differences likely reflect principally pH and temperature effects on the exchange rate.

Discussion

It is readily evident, from the rapid rate of exchange of $^{32}P_i$ with the phosphoenzyme demonstrated in Figures 1 and 2, and from the knowledge that the carboxyl group furnishes the bridge oxygen in the enzyme acyl phosphate (Dahms et al., 1973), that a dynamic reversal of E–P formation from P_i accounts for much and possibly all of the rapid $P_i \rightleftharpoons$ HOH exchange. To assess more carefully whether the $P_i \rightleftharpoons$ E–P exchange rate may be sufficiently rapid to account for all the oxygen exchange, consideration of the steps giving rise to the exchange is necessary.

The formation of E–P from P_i involves as a minimum the steps depicted in eq 1.

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–}P + HOH \qquad (1)$$

Our further discussion is in terms of eq 1, although step one itself could be a multistep process with a conformational change in the E·P_i complex possibly being the actual rate-determining step. Such additional complexity would not alter basic patterns or conclusions presented herein.

The incorporation of water oxygen into P_i occurs by reversal of step 2, and at steady state the total rate of oxygen incorporation is given by k_{-2}(E–P), where the HOH concentration is regarded as incorporated in k_{-2}. From this and the enzyme conservation equation, $(E_t) = (E) + (E\cdot P_i) + (E\text{–}P)$, it is readily shown that the rate of total oxygen exchange between water and E·P_i (E·$P_i \rightleftharpoons$ HOH exchange), designated $v_{ex(total)}$ has the Michaelis–Menten form given in eq 2

$$v_{ex(total)} = \frac{k_{ex}(E_t)(P_i)}{K_m + (P_i)} \qquad (2)$$

where

$$k_{ex} = \frac{k_2 k_{-2}}{k_2 + k_{-2}}$$

and

$$K_m = \frac{k_{-1}k_{-2}}{k_1 k_2 + k_1 k_{-2}}$$

The measured rate of isotope exchange from water into medium P_i will equal the total oxygen exchange rate, k_{-2}(E–P), only if $k_2 \ll k_{-1}$. When k_{-1} becomes significant with respect to k_2, the situation becomes more complicated and the measured rate of oxygen exchange between medium P_i and HOH (the rate estimated from the ^{18}O data) drops below the rate of total oxygen exchange. This is due to the occurrence of reversals of step 2 which do not result in incorporation of water oxygens into medium P_i. As shown in the Appendix, the average number of solvent oxygens incorporated into each P_i which dissociates from the enzyme is:

$$\bar{O} = 4R/(4 + R) \qquad (3)$$

where, $R = k_2/k_{-1}$. The measured rate of oxygen exchange between medium P_i and HOH, designated v_{ex}, can now be expressed as:

$$v_{ex} = \left(\frac{4R}{4 + R}\right) k_{-1}(E\cdot P_i) \qquad (4)$$

Equation 4 along with the enzyme conservation relationship yields exchange kinetics of Michaelis–Menten form with K_m the same as given above and

$$k_{ex} = \left(\frac{4}{4 + R}\right)\frac{k_2 k_{-2}}{k_2 + k_{-2}} \qquad (5)$$

The ratio $4/(4 + R)$ gives the fraction of reversals of step two which result in an incorporation of a water oxygen into medium P_i.

The exchange with sarcoplasmic reticulum ATPase is kinetically quite analogous to that observed with alkaline phosphatase where an enzyme seryl residue is spontaneously phosphorylated by P_i (Schwarz and Lipmann, 1961; Engstrom, 1962; Levine et al., 1969), but rate constants given above as determining k_{ex} and K_m differ from those suggested by Levine et al. (1969) and Reid and Wilson (1971a) for the measured ^{18}O exchange. Their approach used the assumption that no oxygen exchange would accompany the reversible cleavage of E–P to E·P_i.

The rates of the oxygen exchange and $^{32}P_i$ incorporation into E·P_i are both measured under the same dynamic equilibrium conditions, but the equations governing the $^{32}P_i$ distribution must take into consideration the labeling transients as the added $^{32}P_i$ is distributed into E·P_i and E–P. If R is very small, the appearance of $^{32}P_i$ into E–P would increase as in a first-order relationship, asymptotically approaching a maximum, with a $t_{1/2} = 0.693/k_2$, and an initial rate of labeling = k_2(E·P_i). Because k_2(E·P_i) = k_{-2}(E–P), which under these conditions equals the measured $P_i \rightleftharpoons$ HOH exchange rate, the $t_{1/2}$ for ^{32}P labeling of E–P calculated from the oxygen exchange rate should equal the observed $t_{1/2}$.

As R increases and the flux between E·P_i and E–P becomes appreciable compared with that between P_i and E·P_i, the apparent $t_{1/2}$ of labeling of E–P by $^{32}P_i$ would become greater than that predicted from the observed rate of oxygen exchange assuming that only the k_2, k_{-2} step governed the exchange. Our results show an apparent $t_{1/2}$ somewhat greater than predicted on the above assumptions from the $P_i \rightleftharpoons$ HOH exchange rate. It was thus necessary to consider theoretical curves for the expected distribution of $^{32}P_i$ into E·P_i and E–P with time. Such curves may be obtained using approaches described by Sheppard (1962) for isotope distribution in a three-compartment system, the three compartments being equivalent to P_i, E·P, and E–P. For isotope initially present as P_i, the expected isotope distribution with time can be calculated and is a function of the total equilibrium fluxes across steps 1 and 2 (ρ_{12} and ρ_{23}, respectively) and the compartment size. For each value of R, it is possible to calculate an $\bar{O}$ value defined by eq 3. This allows calculation of the fluxes (eq 6 and 7) necessary to yield the measured velocity of isotope exchange.

$$\rho_{12} = V/\bar{O} \qquad (6)$$

$$\rho_{23} = \rho_{12}R \qquad (7)$$

The solid lines in the figures are the calculated theoretical curves for $k_2 \ll k_{-1}$ or k_2/k_{-1} equal or near 0. A much better fit to the experimental data is obtained with a value of k_2/k_{-1} somewhat greater than zero. The dashed lines in the figures are the curves calculated for a k_2/k_{-1} value of 0.08 and with the concentration of E·P_i 3.5-fold larger than that of E–P. The fit is greatly improved and is satisfactory considering the inaccuracy of the data. Also, from estimation of the total number of active sites (see MacLennan and Holland, 1975), a ratio of E·P_i to E–P of 3.5 is in harmony with our data.

From these results it is apparent that the rate of flux between medium P_i and E–P is sufficiently rapid to account for most

and quite likely all of the oxygen exchange. A catalytic capacity for a $P_i \rightleftharpoons HOH$ exchange has also been observed with intestinal (Stein and Koshland, 1952) or *E. coli* alkaline phosphatase (Schwarz and Lipmann, 1961), inorganic pyrophosphatase (Cohn, 1958), myosin ATPase (Levy et al., 1960; Dempsey et al., 1963), and mitochondria or mitochondrial membranes capable of oxidative phosphorylation (Cohn, 1953; Mitchell et al., 1967). With *E. coli* alkaline phosphatase (Reid and Wilson, 1971a,b) and with myosin ATPase (Bagshaw et al., 1975), the dynamic reversal of hydrolytic cleavage of the O-P bond appears to be sufficiently rapid to account for the oxygen exchange. To these must be added now the sarcoplasmic reticulum ATPase. Also, with inorganic pyrophosphatase (Jansen and Boyer, unpublished) and mitochondrial membranes (Boyer et al., 1974), reversible formation from P_i of bound PP_i or bound ATP, respectively, has been observed. For all these enzymes it is thus unnecessary at present to postulate an exchange involving a formation of a pentacovalent adduct of the P_i of the phosphoryl group with water. Stein and Koshland (1951) argued against a pentacovalent intermediate on the basis of lack of ^{18}O incorporation into the unhydrolyzed phosphate during phosphatase-catalyzed hydrolysis. Such a possible pentacovalent intermediate has been mentioned for pyrophosphatase exchange (Cohn, 1958) and has been suggested to have a role in oxidative phosphorylation by mitochondria (Korman and McLick, 1973).

It should be emphasized, however, that none of the data eliminates the possibility of additional intermediates in exchanges ccurring by dynamic reversal of hydrolysis of the phosphorylated intermediates E-P, E·PP_i, or E·ATP. For example the sequence given by eq 1 might be expanded to include formation of a bound metaphosphate as depicted by eq 8.

$$E \cdot P_i \overset{HOH}{\longleftrightarrow} E \cdot \text{metaphosphate} \longleftrightarrow E\text{-}P \qquad (8)$$

Our results would mean that any such bound metaphosphate has a rapid flux with E-P.

The rapid flux between P_i and E-P at pH near 6, where the enzyme is maximally phosphorylated by P_i, establishes the reversible cleavage of the acyl phosphate as a rapid and perhaps the most rapid reaction catalyzed by the enzyme. It is evident that the enzyme active site has a remarkable kinetic as well as thermodynamic capacity to favor acyl phosphate formation from P_i.

Acknowledgment

The technical assistance of Lynne Arnold in performing ^{18}O analyses is gratefully acknowledged.

Appendix: Derivation of Equation 3 for $\bar{O}$

On the basis of probability considerations, the average number of oxygens exchanged before a phosphate is released from the enzyme, $\bar{O}$, is:

$$\bar{O} = \sum_{i=0}^{\infty} (1 - P_c)P_c{}^i 4[1 - (\tfrac{3}{4})^i] \qquad (9)$$

where P_c is the partitioning coefficient, $k_2/(k_2 + k_{-1})$, expressing the probability that E·P_i, once formed, will go on to form E-P + H_2O. The term $(1 - P_c)(P_c{}^i)$ is the probability of a P_i being released after i reversals of the formation of E-P. The term $4[1 - (\tfrac{3}{4})^i]$ is the average number of oxygens exchanged for each ith integral number of reversals of the formation of E-P and is equal to

$$\sum_{j=1}^{i} (\tfrac{3}{4})^{j-1}$$

Equation 9 is thus a summation over all reversals of the probability of a given reversal multiplied by the oxygen exchange for that number of reversals.

Now:

$$\bar{O} = 4\sum_{i=0}^{\infty} (P_c{}^i - P_c{}^{i+1})[1 - (\tfrac{3}{4})^i]$$

$$\bar{O} = 4\{[(1 - \tfrac{3}{4})]P_c{}^1 - (1 - \tfrac{3}{4})P_c{}^2 + [1 - (\tfrac{3}{4})^2]P_c{}^2 - [1 - (\tfrac{3}{4})^2]P_c{}^3 + [1 - (\tfrac{3}{4})^3]P_c{}^3 - [1 - (\tfrac{3}{4})^3]P_c{}^4 + \ldots\}$$

$$\bar{O} = 4\sum_{i=1}^{\infty} [(\tfrac{3}{4})^{i-1} - (\tfrac{3}{4})^i]P_c{}^i$$

$$\bar{O} = \tfrac{4}{3}\sum_{i=1}^{\infty} (\tfrac{3}{4}P_c)^i$$

$$\bar{O} = \tfrac{4}{3}\left[\frac{(\tfrac{3}{4}P_c)}{1 - \tfrac{3}{4}P_c}\right]; \quad \sum_{i=1}^{\infty} b^i = \frac{b}{1-b} \text{ for } b < 1$$

$$\bar{O} = \frac{4P_c}{4 - 3P_c}$$

$$\bar{O} = \frac{4R}{4 + R}; \quad R = \frac{k_2}{k_{-1}}; \quad P_c = \frac{R}{R + 1}$$

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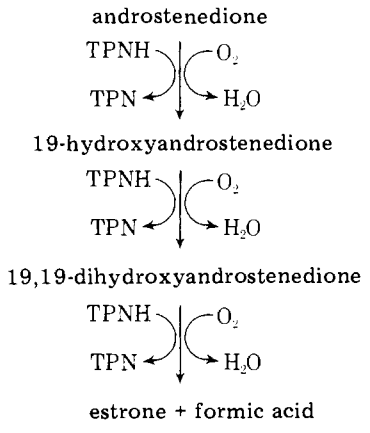
Aromatization of Δ^4-Androstene-3,17-dione, 19-Hydroxy-Δ^4-androstene-3,17-dione, and 19-Oxo-Δ^4-androstene-3,17-dione at a Common Catalytic Site in Human Placental Microsomes†

William G. Kelly,* Dianne Judd, and Ann Stolee

ABSTRACT: Estrogen is believed to be biosynthesized from androstenedione in placental microsomes by a multienzyme pathway in which 19-hydroxyandrostenedione and 19-oxoandrostenedione (or the hydrated form) are obligatory intermediates. However, both 19-hydroxyandrostenedione and 19-oxoandrostenedione competitively inhibited aromatization of androstenedione, and all three steroids were shown to be mutually competitive. 19-Hydroxyandrostenedione and 19-oxoandrostenedione also competed with androstenedione for binding sites in the microsomes at 4 °C. In confirmation of the work of Hollander (Hollander, N. (1962), *Endocrinology 71,* 723–728), and of Osawa and Shibata (Osawa, Y., and Shibata, K., (1973), Abstracts of the 55th Meeting of the Endocrine Society, Abstract 116) when androstenedione and 19-hydroxyandrostenedione were incubated together, both were converted to estrogen, but little androstenedione was converted to 19-hydroxyandrostenedione. Considered together, these results are incompatible with the multienzyme pathway. Rather, these results may be explained by aromatization of androstenedione at a single catalytic site via enzyme-bound transition states. Both proposed intermediates are, according to this view, by-products which can also be aromatized.

The conversion of androstenedione to estrone in human placental microsomes is believed to proceed according to the multistep pathway in Scheme I.

Scheme I



19-Hydroxyandrostenedione and 19,19-dihydroxyandrostenedione (or the dehydrated form, 19-oxoandrostenedione) are considered obligatory intermediates in that each is the product of one enzymatic reaction and the substrate for the next. The pathway as formulated requires three enzymes together with an electron transport system, all of which are associated with the microsomal membranes. The evidence in support of this multistep, multienzyme pathway has recently been reviewed by Engel (1973) and is based on the demonstration of the formation of each intermediate prior to formation of estrogen and on the facile conversion of both intermediates to estrogen. Further support for this pathway is provided by the determination that 3 mol of TPNH[1] and 3 mol of oxygen are consumed for each mole of estrogen produced (Thompson and Siiteri, 1974a).

In 1962, Hollander reported that, when she incubated radiolabeled androstenedione and unlabeled 19-hydroxyandrostenedione with placental microsomes, the estrogen isolated had a higher specific activity than did 19-hydroxyandrostenedione. She concluded that 19-hydroxyandrostenedione could not be an obligatory intermediate. In 1973, Osawa and Shibata reported to the Endocrine Society that they had obtained similar results from a similar experiment.

The work reported here shows that both 19-hydroxyandrostenedione and 19-oxoandrostenedione are competitive inhibitors of the aromatization of androstenedione and that both of these steroids compete with androstenedione for common binding sites in placental microsomes under conditions where formation of estrogen is negligible. These results strongly support the hypothesis that the conversion of androstenedione to estrogen proceeds by a concerted mechanism at a single catalytic site without the dissociation of intermediates,

† From the Departments of Obstetrics and Gynecology, and Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455. *Received May 3, 1976.* This work was supported by Research Grant AM 17570 from the National Institutes of Health.

[1] Abbreviations used are: TPNH, reduced triphosphopyridine nucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Butyl PBD, 2(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole.