PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 176–181 (1974)

# Isolated Adrenal Cortex Cells: $ACTH_{4-23}(NH_2)$, $ACTH_{5-24}$, $ACTH_{6-24}$ and $ACTH_{7-23}(NH_2)$; Cyclic AMP and Corticosterone Production[1] (37772)

GEORGE SAYERS, STEVEN SEELIG,[2] SATYENDRA KUMAR, GEORG KARLAGANIS, ROBERT SCHWYZER, AND MASAHIKO FUJINO

*Department of Physiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106; Institute of Molecular Biology and Biophysics, Swiss Federal Institute of Technology, 8049 Zurich, Switzerland; and Chemical Research Laboratories, Research and Development Division, Takeda Chemical Industries, Ltd., Osaka, Japan*

The active center of the ACTH molecule appears to be located near its N terminal. The peptide $ACTH_{1-10}$ stimulates corticosterone production *in vivo* (1) and *in vitro* (2). Furthermore, $ACTH_{4-23}(NH_2)$ exhibits a high degree of activity, $ACTH_{5-23}(NH_2)$ and $ACTH_{6-24}$ exhibit low degrees of activity and $ACTH_{7-23}(NH_2)$ is inactive when tested *in vivo* (3). In order to further define the roles of the amino acids in the sequence $Met^4\times$ $Glu^5\times$ $His^6\times$ $Phe^7\times$ $Arg^8\times$ $Trp^9\times$ $Gly^{10}$ we have examined the responses of suspensions of isolated adrenal cortex cells of the rat to the additions of $ACTH_{4-23}(NH_2)$, $ACTH_{5-24}$, $ACTH_{6-24}$ and $ACTH_{7-23}(NH_2)$. Complete log concentration response curves for cyclic AMP (cAMP) and for corticosterone production are displayed. Of some interest is the finding that certain analogues may be partial agonists for cAMP production and at the same time full agonists for corticosterone production.

*Methods and Materials.* Suspensions of cells were prepared from the fasciculata-reticularis region of 40 rat adrenals according to the method of Sayers, Swallow and Giordano (4). Aliquots of the suspension, together with vehicle or with vehicle to which had been added ACTH, were incubated in an atmosphere of 95% $O_2$:5% $CO_2$ at 37°. At the end of 60 min of incubation, methylene chloride was added; an aliquot of the methylene chloride phase was analyzed for corticosterone (5) and an aliquot of the aqueous phase was analyzed for cyclic [8-$^{14}$C]AMP by the method of Kuo and De Renzo (6) as adapted for use in isolated adrenal cortex cell preparations by Beall and Sayers (7). In all cases, results are expressed as net cAMP production and net corticosterone production; quantities of cAMP and of corticosterone in aliquots to which no ACTH was added (blanks) have been subtracted.

The peptides $ACTH_{4-23}(NH_2)$, $ACTH_{6-24}$ and $ACTH_{7-23}(NH_2)$ were synthesized and purified in the laboratory of Masahiko Fujino by methods previously described (3). $ACTH_{5-24}$ was synthesized and purified in the laboratory of Robert Schwyzer. $ACTH_{1-24}$ was provided as Synacthen by Dr. W. Rittel, CIBA-GEIGY AG, Basel, Switzerland, and as Cortrosyn by Dr. H. Strade,

[1] The following abbreviations of amino acids and peptides are used [IUPAC-IUB Commission on Biochemical Nomenclature, *Arch. Biochem. Biophys.* **150**, 1 (1972)]: $ACTH_{1-24}$ = corticotropin-(1–24)-tetracosapeptide; $ACTH_{4-23}(NH_2)$ = corticotropin-(4–23)-eicosapeptide amide; $ACTH_{5-24}$ = corticotropin-(5-25-eicospeptide; $ACTH_{6-24}$ = corticotropin-(6–24)-nanodecapeptide and $ACTH_{7-23}(NH_2)$ = corticotropin-(7–23)-heptadecapeptide amide. This work was supported by NSF Grant GB-27426 and USPHS Training Grant 5 T01 GM 00899.

[2] Predoctoral Fellow, USPHS Training Grant 5 T01 GM00899.

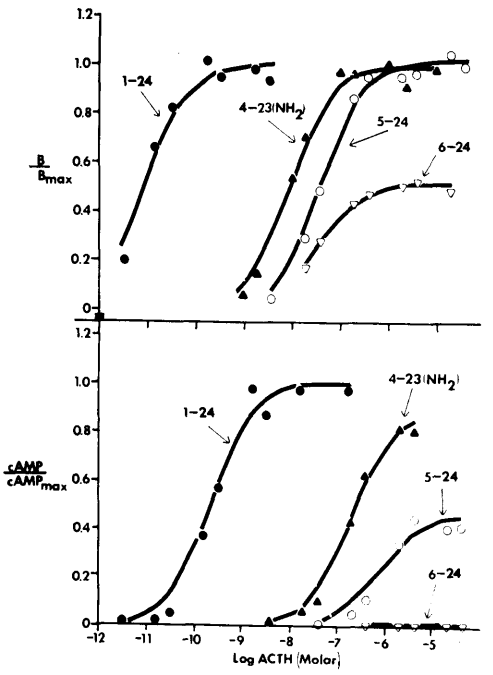


FIG. 1. Log concentration curves for cAMP production and corticosterone production by aliquots of a suspension of isolated adrenal cortex cells in response to $ACTH_{1-24}$ (●), $ACTH_{4-23}(NH_2)$ (▲), $ACTH_{5-24}$ (○) and $ACTH_{6-24}$ (△). The points are means of analyses on two aliquots of cell suspension. The lines represent least square fits of cAMP or corticosterone production by a nonlinear method (8) to the equation $V/\alpha V_{max} = A/(A + A_{50})$ where $V$ is either the rate of cAMP or corticosterone production; $V_{max}$ is either the maximum rate of cAMP production ($cAMP_{max}$) or corticosterone production ($B_{max}$) induced by $ACTH_{1-24}$; $\alpha$ is the intrinsic activity (the maximum rate of cAMP production for analogue/$cAMP_{max}$ or the maximum rate of corticosterone production for analogue/$B_{max}$; $A$ and $A_{50}$ are the concentration and the concentration of analogue required to induce $\frac{1}{2}\ \alpha V_{max}$, respectively. The abscissa represents log molar concentration of ACTH analogue; the top ordinate represents micrograms of corticosterone per 60 min divided by $B_{max}$; the bottom ordinate represents cpm cyclic[8-$^{14}$C]AMP/60 min divided by $cAMP_{max}$. The data are compiled from experiments on three cell suspensions carried out on different days. $ACTH_{1-24}$ was employed as a standard of reference in each experiment. Since $cAMP_{max}$ and $B_{max}$ varied with the cell suspension, $cAMP_{max}$ and $B_{max}$ were determined for each cell suspension by the nonlinear least squares fit of the data for various concentrations of $ACTH_{1-24}$. $B_{max}$ values for the suspensions were 1.23, 0.630 and 0.928 $\mu$g/60 min; $cAMP_{max}$ values were 1314, 628 and 1264 cmp/60 min. Blanks for corticosterone production equaled 0.038, 0.034 and 0.038 $\mu$g/60 min; for cAMP production, 6, 7 and 9 cpm/60 min.

Organon Ltd., West Orange, NJ.

*Results.* $ACTH_{1-24}$, $ACTH_{4-23}(NH_2)$, $ACTH_{5-24}$ and $ACTH_{6-24}$ induce an increase in cAMP and corticosterone production when added to suspensions of isolated adrenal cortex cells. Log concentration curves for corticosterone production and cAMP production are presented in Fig. 1. $ACTH_{4-23}(NH_2)$, $ACTH_{5-24}$ and $ACTH_{6-24}$ have intrinsic activities for corticosterone production ($\alpha$ = the maximum rate of corticosterone production of an analogue divided by $B_{max}$, the maximum rate of corticosterone production for $ACTH_{1-24}$) equal to 1.0, 1.0, and 0.4, respectively, and intrinsic activities for cAMP production ($\alpha$ = the maximum rate of cAMP production for an analogue divided by $cAMP_{max}$, the maximum rate of cAMP production for $ACTH_{1-24}$) equal to 0.86, 0.45, and 0.01, respectively.

As the N-terminus is shortened, there is a progressive displacement of the log concentration curves away from the log concentration curves for $ACTH_{1-24}$. The value of $A_{50}$ (Table I), the dose required to induce $\frac{1}{2}\ \alpha$ $cAMP_{max}$ or $\frac{1}{2}\ \alpha\ B_{max}$, is a quantitative expression of this displacement and reflects the dissociation constant of the analogue for the receptor. For $ACTH_{1-24}$, $ACTH_{4-23}(NH_2)$ and $ACTH_{5-24}$ the value of $A_{50}$ for cAMP production is approximately 40 times greater than the value of $A_{50}$ for corticosterone production. The low cAMP intrinsic activity for $ACTH_{6-24}$ precluded the determination of its $A_{50}$ from its ability to induce a response.

A value of $A_{50}$ for $ACTH_{6-24}$ was determined by its ability to inhibit cAMP and corticosterone production induced by $ACTH_{1-24}$. Figure 2 presents log concentration curves for $ACTH_{1-24}$ and $ACTH_{6-24}$ acting alone (panel A) and in combination (panels B and C). Increasing concentrations of $ACTH_{6-24}$ decreased the rate of cAMP and corticosterone production induced by $1.75 \times 10^{-8}$ $M$ $ACTH_{1-24}$ (Fig. 2B). Increasing concentrations of $ACTH_{1-24}$ in-

TABLE I.

| | Corticosterone production | | Cyclic AMP production | |
|---|---|---|---|---|
| | $A_{50}$[a] | $\alpha$[b] | $A_{50}$[a] | $\alpha$[b] |
| $ACTH_{1-24}$ | $2.6 \times 10^{-11}$ | 1 | $1.03 \times 10^{-9}$ | 1 |
| $ACTH_{4-23}(NH_2)$ | $3.28 \times 10^{-9}$ | 1 | $1.65 \times 10^{-7}$ | .88 |
| $ACTH_{5-24}$ | $1.2 \times 10^{-7}$ | 1 | $3.68 \times 10^{-6}$ | .45 |
| $ACTH_{6-24}$ | $8.88 \times 10^{-8}$ | 0.4 | $1.39 \times 10^{-7}$[c] | .01 |
| $ACTH_{7-23}(NH_2)$ | $1.24 \times 10^{-7}$[c] | 0 | $7.05 \times 10^{-8}$[c] | 0 |

[a]Apparent dissociation constant estimated from the molar concentration of analogue required to induce 1/2 $\alpha B_{max}$ or 1/2 $\alpha$ $cAMP_{max}$.

[b]The intrinsic activity estimated from the maximum corticosterone production induced by the analogue divided by the maximum corticosterone production induced by $ACTH_{1-24}$ or the maximum cAMP production induced by the analogue divided by the maximum cAMP production induced by $ACTH_{1-24}$.

[c]Apparent dissociation constant estimated by capacity of analogue to inhibit the response induced by $ACTH_{1-24}$.

creased the rate of cAMP and corticosterone production induced by $4.35 \times 10^{-7}$ *M* $ACTH_{6-24}$ (Fig. 2C).

$ACTH_{7-23}(NH_2)$ was biologically inactive at a concentration of $4.8 \times 10^{-4}$ *M*. The possibility existed that $ACTH_{7-23}(NH_2)$ retained an affinity for the receptor, yet failed to activate the receptor. To test this possibility $ACTH_{7-23}(NH_2)$ was added in combination with $ACTH_{1-24}$ (Fig. 3). Concentrations of $0.58 \times 10^{-6}$ and $2.32 \times 10^{-6}$ *M* displaced to the right the log concentration curve for $ACTH_{1-24}$. Increasing doses of $ACTH_{1-24}$ reversed the inhibitory action of $ACTH_{7-23}(NH_2)$.

*Discussion.* On the basis of corticosterone production in the isolated cell system, $ACTH_{4-23}(NH_2)$ and $ACTH_{5-24}$ are *agonists* (intrinsic activities equal to 1.0). $ACTH_{6-24}$ is active but induces a maximum less than $B_{max}$; $ACTH_{7-23}(NH_2)$ is inactive (Table I). These findings confirm and extend the *in vivo* observations of Fujino, Hatanaka and Nishimira (3) and support their suggestion that the sequence $His^6 \times Phe^7 \times Arg^8 \times Trp^9 \times Gly^{10}$ is important in endowing the hormone with steroidogenic activity.

On the basis of cAMP production, $ACTH_{4-23}(NH_2)$ and $ACTH_{5-24}$ are partial agonists (intrinsic activities of 0.86 and 0.45, respectively) and $ACTH_{6-24}$ is practically inactive (Table I), findings which suggest that $Met^4 \times Glu^5$ is important in endowing the hormone with the capacity to induce cAMP production.

These differences in the structural requirements for maximal production of cAMP and of corticosterone suggest a dual receptor model for the adrenal cortical cell. Steroidogenesis is the result of the interaction of ACTH with a "*B*" receptor. Cyclic AMP production is the result of the interaction of ACTH with a "*C*" receptor. The model accounts for the fact that $ACTH_{1-24}$ at low concentrations induces a highly significant increase in the rate of steroidogenesis without a measurable increase in cAMP accumulation. The apparent dissociation constant for the *B* receptor is about 1/40th that of the *C* receptor ($A_{50}$ for $ACTH_{1-24}$ are $2.6 \times 10^{-11}$ vs $1.03 \times 10^{-9}$ *M* for corticosterone and cAMP, respectively, see Table I). Compared to $ACTH_{1-24}$, $ACTH_{5-24}$ retains full capacity to excite the *B* receptor but exhibits reduced capacity to excite the *C* receptor. AC $TH_{6-24}$ exhibits a slightly reduced efficiency with respect to the *B* receptor but is practically inactive with respect to the *C* receptor. This dual receptor model leaves open the question as to the mediator role of cAMP in steroidogenesis. An unknown factor or factors may be involved in the events between the interaction of ACTH with a *B* receptor and increased production of corticosterone or, if cAMP is involved, the nucleotide is produced in a special compartment and in a quantity too small to be detected. According to the dual receptor thesis, the measurable cAMP produced in response to activation of the *C* receptor is not directly related to corticos-

terone biosynthesis. Moyle, Kong and Ramachandran (9) have proposed that there are two receptors for ACTH in the adrenal cell population which may be in the same cell or in different cell types.

A model which invokes a single receptor site and which is based on the generally accepted thesis that cAMP mediates the steroidogenic action of ACTH has been proposed by Seelig and Sayers (10). The observation that $ACTH_{7-23}(NH_2)$ induces the same shift to the right of the $ACTH_{1-24}$ log concentration curves for cAMP and corticosterone production is compatible with a single receptor mechanism. The higher apparent dissociation constant for cAMP production, relative to that for corticosterone production, is ascribed to receptor reserve (10) in the system. $B_{max}$ is attained when less than 20% of the total receptor population is engaged by $ACTH_{1-24}$, 50% of $B_{max}$, when about 1% of the receptor population is engaged. These

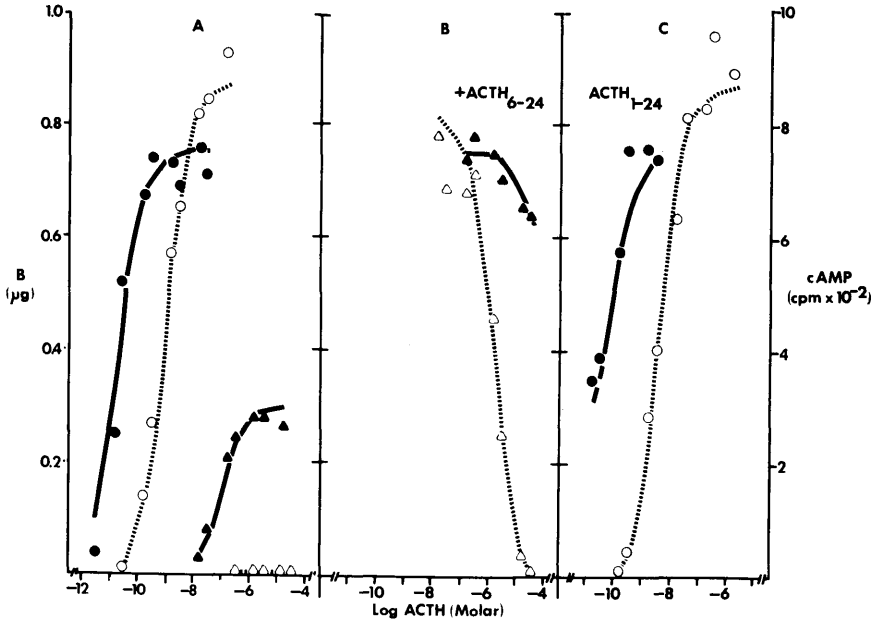


FIG. 2. Log concentration curves for corticosterone production (—) and cAMP production (- - - -) by aliquots of a single suspension of isolated adrenal cortex cells in response to $ACTH_{1-24}$ alone (● or ○) and $ACTH_{6-24}$ alone (▲ or △) (panel A); in response to increasing concentrations of $ACTH_{6-24}$ (▲ or △) in combination with $1.75 \times 10^{-8}$ $M$ $ACTH_{1-24}$ (panel B); and in response to increasing concentrations of $ACTH_{1-24}$ (● or ○) in combination with $4.35 \times 10^{-7}$ $M$ $ACTH_{6-24}$ (panel C). The points are means of analyses on two aliquots of cell suspension. The lines represent nonlinear least square fits of corticosterone and cAMP production to the equation $V/V_{max} = \{\alpha A/[A + (1 + P/P_{50})A_{50}]\} + \{[\beta P/P + (1 + A/A_{50})P_{50}]\}$ where $\alpha$, $A$ and $A_{50}$ are the intrinsic activity, concentration and concentration required to induce ½ $\alpha V_{max}$ for $ACTH_{1-24}$, respectively; and where $\beta$, $P$ and $P_{50}$ are the intrinsic activity, concentration and concentration required to induce ½ $\beta V_{max}$ for $ACTH_{6-24}$, respectively. $V$ and $V_{max}$ are defined in the legend to Fig. 1. In the case of panel A, for $ACTH_{1-24}$ acting alone, $P = 0$ and for $ACTH_{6-24}$ acting alone, $A = 0$. The abscissa for panel A represents log molar concentration of the two ACTH acting alone; the abscissa for panel B represents log molar concentration of $ACTH_{6-24}$ acting in combination with $ACTH_{1-24}$; the abscissa for panel C represents log molar concentration of $ACTH_{1-24}$ acting in combination with $ACTH_{6-24}$. The ordinates represent µg corticosterone/60 min and cpm cyclic[$8^{-14}$C]AMP/60 min. Blanks for corticosterone production equaled 0.036 and 0.036 µg/60 min; blanks for cAMP production equalled 10 and 12 cpm/60 min. A concentration of $4.35 \times 10^{-5}$ $M$ $ACTH_{6-24}$ induced a cAMP response of 4 cpm above the blank.

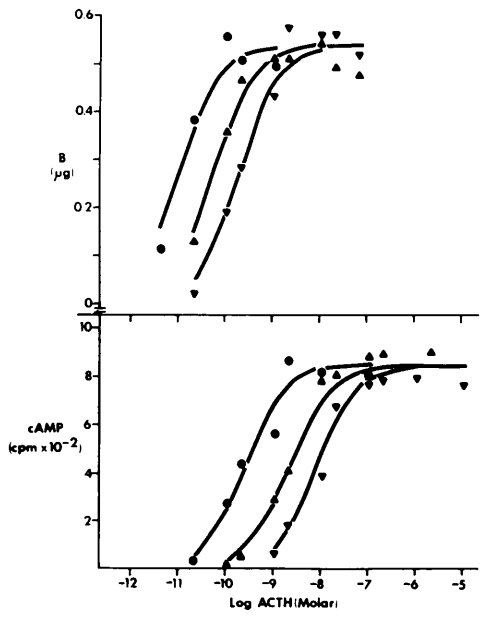


FIG. 3. Log concentration curves for cAMP production and corticosterone production by aliquots of a suspension of isolated adrenal cortex cells in response to $ACTH_{1-24}$ alone (●), in combination with $0.58 \times 10^{-6}$ *M* $ACTH_{7-23}(NH_2)$ (▲), and with $2.32 \times 10^{-6}$ *M* $ACTH_{7-23}(NH_2)$ (▲). The points are the means of analyses on two aliquots of cell suspension; the lines represent nonlinear least square fits to the equation $V/_{\alpha}V_{max} = A/A + (1 + I/I_{50})\ A_{50}$ where $I$ is the concentration of $ACTH_{7-23}(NH_2)$ and $I_{50}$ is the concentration of $ACTH_{7-23}(NH_2)$ required to shift the log concentration curve of $ACTH_{1-24}$ by a factor of 2 to the right. The $\alpha$, $V$, $V_{max}$, $A$ and $A_{50}$ are defined in the legend to Fig. 1. The abscissa represents log molar concentration of $ACTH_{1-24}$; the top ordinate is μg corticosterone production/60 min and the bottom ordinate is cmp cyclic[$8^{-14}$C]AMP/60 min. The maximum rate of cAMP production and corticosterone production induced by $ACTH_{1-24}$ equaled 848 cpm/60 min and 0.54 μg/60 min, respectively. Blanks for cAMP production equaled 8 and 10 cpm/60 min; blanks for corticosterone production equaled 0.04 and 0.04 μg/60 min.

relations are revealed by examination of the log concentration curves for $ACTH_{1-24}$ displayed in Fig. 1. $B_{max}$ is attained at a concentration of $ACTH_{1-24}$ which induces about 20% of $cAMP_{max}$ and 50% $B_{max}$ at a concentration of $ACTH_{1-24}$ which induces only 1% of $cAMP_{max}$. $ACTH_{5-24}$, although 45% as efficient as $ACTH_{1-24}$ in activating the receptors, induces $B_{max}$ by engaging a larger fraction (about 40%) of the receptor population. $ACTH_{6-24}$, by engaging the total receptor population, is able to induce a maximum rate of corticosterone production equal to 0.4 that of $ACTH_{1-24}$. The receptor reserve model is based on the assumption that low concentrations of $ACTH_{1-24}$ induce significant steroidogenesis (less than 50% $B_{max}$) with associated small, but presently nondetectable changes in cAMP production. Two of the authors (S. S. and G. S.) are now engaged in experiments designed to test the validity of this assumption.

*Summary.* Suspensions of isolated adrenal cortex cells of the rat respond to the addition of $ACTH_{1-24}$ and related peptides with production of cAMP and corticosterone. A concentration of $ACTH_{1-24}$ which results in less than 20% of maximum cAMP production induces near-maximum corticosterone production; a concentration which results in 1–3% cAMP production induces 50% of maximum corticosterone production. $ACTH_{4-23}(NH_2)$ and $ACTH_{5-24}$ are less potent than AC $TH_{1-24}$, but induce the same maximum corticosterone production ($B_{max}$) as $ACTH_{1-24}$; $ACTH_{6-24}$ induces a maximum equal to 0.4 of $B_{max}$; $ACTH_{7-23}(NH_2)$ is inactive. AC $TH_{4-23}(NH_2)$, $ACTH_{5-24}$ and $ACTH_{6-24}$ induce 0.86, 0.45 and 0.01 of the maximum cAMP production characteristic of AC $TH_{1-24}$, respectively; $ACTH_{7-23}(NH_2)$ is inactive. $ACTH_{7-23}(NH_2)$ is a competitive antagonist and shifts the $ACTH_{1-24}$ log concentration curves for cyclic AMP and for corticosterone to the same degree. These observations are discussed in terms of a dual receptor model and in terms of a single site-receptor reserve model.

The authors thank Mary Vegh for excellent technical assistance.

1. Ney, R. L., Ogata, E., Schimizu, N., Nicholson, W. E., and Liddle, G. W., Proc. Sec. Int. Congr. Endocrinol. Part II, 1184 (1964).

2. Schwyzer, R., Schiller, P., Seelig, S., and Sayers, G., FEBS (Fed. Eur. Biochem. Soc.) Lett. 19, 229 (1971).

3. Fujino, M., Hatanaka, C., and Nishimira, O., Chem. Pharm. Bull. 19, 1066 (1971).

4. Sayers, G., Swallow, R. L., and Giordano, N. D., Endocrinology 88, 1063 (1971).

5. Silber, R. H., Busch, R. D., and Oslapas, R., Clin. Chem. 4, 278 (1958).
6. Kuo, J. F., and De Renzo, E. C., J. Biol. Chem. **244**, 2252 (1969).
7. Beall, R. J., and Sayers, G., Arch. Biochem. Biophys. **148**, 70 (1972).
8. Cleland, W. W., Advan. Enzymol. Relat. Areas Mol. Biol. **29**, 1 (1967).
9. Moyle, W. R., Kong, Y. C., and Ramachandran, J., J. Biol. Chem. **248**, 2409 (1973).
10. Seelig, S., and Sayers, G., Arch. Biochem. Biophys. **154**, 230 (1973).

Received July 17, 1973. P.S.E.B.M., 1974, Vol. 145.