Enzymatic Coupling of Isoniazid to Proteins*

L. Lorand,† L. K. Campbell, and B. J. Robertson

ABSTRACT: Isonicotinic acid hydrazide (isoniazid) acts as an amine substrate (or inhibitor) in transamidating reactions catalyzed by liver transglutaminase. Using [^{14}C]isoniazid, coupling to benzyloxycarbonyl-L-glutaminylglycine, a known acceptor substrate of the enzyme was shown to occur. Transglutaminase also brought about the covalent incorporation of isoniazid into a variety of protein acceptors. Specifically, enzymatic attachment of the compound to α-casein, β-lactoglobulin, serum α-globulin, and albumin were demonstrated. Moreover, isoniazid becomes incorporated into the fibrin clot in plasma under conditions which enable fibrin-dependent transamidation to take place. The enzymatic covalent modification of proteins with isoniazid may be relevant in explaining some of the biological effects of this tuberculostatic drug, including the occurrence of autoimmunization.

Isonicotinic acid hydrazide (isoniazid), an important tuberculostatic agent, is one of the most commonly used drugs (see, *e.g.*, Goodman and Gilman, 1965). However, the molecular basis of its effectiveness still remains to be explained. The amino group in the compound appears to be essential for activity because alkylation, acetylation, and condensation with aldehydes lead to a decrease of pharmacological potency. Transacetylation from acetylcoenzyme A and hydrolysis are thought to be the major pathways for the metabolic transformations of the drug in man, with acetylisoniazid and isonicotinic acid as the main excretion products. Untoward side effects during treatment range from apparent pyridoxine deficiency and nervous system dysfunctions to allergic manifestations.

We reported earlier (Lorand and Jacobsen, 1967) that isoniazid inhibited the cross-linking of fibrin, which is a transamidase-catalyzed step in blood coagulation and normally leads to the formation of inter-fibrin γ-glutamyl-ϵ-lysine bonds (Lorand *et al.*, 1968a,b; Matacic and Loewy, 1968; Pisano *et al.*, 1968). In addition, isoniazid could also be shown (Lorand and Campbell, 1971; Lorand *et al.*, 1971) to inhibit reactions by some related enzymes, such as liver transglutaminase (Clarke *et al.*, 1959; Folk and Cole, 1966) and muscle transpeptidase (Myhrman and Bruner-Lorand, 1970).

Since this type of enzyme activity, though of unknown biological significance so far, is widely distributed in cells, it was deemed to be of importance to investigate the relation of isoniazid to transamidases in greater detail. The present paper provides a demonstration that, by virtue of being a substrate for these enzymes, isoniazid is covalently incorporated into a variety of proteins.

Materials and Methods

Liver transglutaminase was prepared and assayed according to the methods of Folk and Cole (1966). The enzyme solution was stored at $-10°$ in 5 mM Tris-hydrochloride buffer (pH 7.5), containing 2 mM EDTA.

α-Casein was purchased from Mann Research Laboratories, β-lactoglobulin from Pentex. They were dissolved in 0.05 M Tris-hydrochloride buffer (pH 7.5); concentrations were calculated on the basis of the weight of lyophilized proteins added.

Human plasma protein fractions were obtained from Pentex and were made up in the above Tris buffer, followed by dialysis overnight against the same at 4°. If cloudy, the solutions were clarified by passage through a GS 0.22 μ Millipore filter before use. Concentrations for these fractions are based on an assumed absorbancy of 10 at 280 nm for 1% solutions.

Serum was obtained by adding 0.1 ml of bovine thrombin (2 units; Parke-Davis) to 2 ml of citrated human plasma, followed by removal of the clot after 3-hr standing at room temperature.

[^{14}C]Isoniazid (carbonyl-[^{14}C]isonicotinic acid hydrazide) was purchased from Amersham-Searle with specific activities of 9.85 and 5.0 mCi per mmole. The isotope was dissolved in 0.05 M Tris-hydrochloride at pH 7.5 and was stored frozen. Nonradioactive isoniazid was obtained from Eli Lilly; histamine dihydrochloride from Mann Laboratories. Hydralazine (1-hydrazinonaphthalazine) was received from Ciba.

Apart from enzyme and substrate concentrations specified in individual figure legends, the reaction mixtures typically contained 20 mM of dithiothreitol, calcium chloride, and 50 mM Tris-hydrochloride at pH 7.5. Reactions were performed at 37° (Lab-Line block) in total volumes of 75 μl, using 5 × 25 mm Pyrex tubes. Samples of 2 μl were withdrawn at various times and were analyzed by the thin-layer cellulose chromatographic procedure of Lorand and Campbell (1971), using precoated Eastman plastic sheets with 1% (v/v) pyridine–acetic acid buffer (pH 5.4) as developer. This provided an excellent separation of the protein-bound isoniazid from the unreacted compound, with the former remaining at the origin and the latter moving near the solvent front at a R_F of about 0.95. Following chromatographic separation, 1-cm^2 sections were cut around the original sites of application which were then immersed in 15 ml of Bray's scintillation fluid for isotope counting.

For experiments shown in Figures 1 and 2, benzyloxycarbonyl-L-glutaminlyglycine (Folk and Cole, 1966) was obtained from Cyclo Corp. and the fluorescent "monodansylcadaverine" [*i.e.*, *N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide] (Lorand *et al.*, 1968a) from Kabi, Stockholm. Separation of the enzymatically formed coupling prod-

* From the Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received September 23, 1971.* This work was aided by a U. S. Public Health Service Research Career award (5-K6-HE-3512) and by grants from the National Institutes of Health (HE-02212, GM 17936, and 5TI-GM 626) and the American Heart Association.

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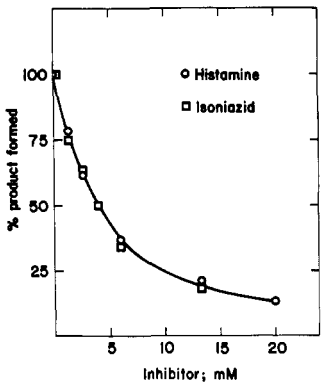


FIGURE 1: Inhibitory effect of isoniazid on the transglutaminase-catalyzed coupling reaction between benzyloxycarbonyl-L-glutaminylglycine and monodansylcadaverine. Concentration of the former substrate was 30 mM, that of the latter 0.2 mM. Transglutaminase activity was 0.13 unit/ml. The effect of isoniazid (□) was compared with histamine (○), a known inhibitor of the reaction. Initial concentrations of both isoniazid and histamine are shown on the abscissa.

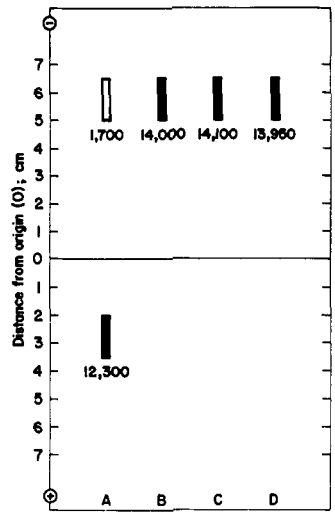


FIGURE 2: Transglutaminase-catalyzed coupling of [^{14}C]isoniazid to benzyloxycarbonyl-L-glutaminylglycine. Concentrations of these substrates were 0.7 mM (9.85 mCi/mmole) and 30 mM, respectively. Enzyme activity was 0.64 unit/ml. After 3-hr reaction (37°), electrophoresis was performed as described in the section on Methods, followed by isotope counting. The bars represent the approximate spread of radioactive spots in the direction of migration and the figures indicate the total counts per minute. (A) Complete system; (B) transglutaminase plus isoniazid, without benzyloxycarbonyl-L-glutaminylglycine; (C) both substrates, but no enzyme; (D) isoniazid alone.

uct between these two substrates was carried out by thin-layer chromatography on a Brinkman MN-polygram polyamide-11 plastic sheet as described by Lorand and Campbell (1971). The extent of the reaction was monitored by scanning the plate for fluorescence as given in this reference.

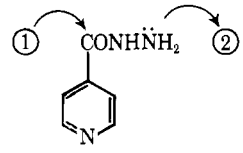
With plasma, two sets of mixtures were prepared. Both contained 40 μl of citrated platelet-poor human plasma and 10 μl of the [^{14}C]isoniazid solution (10 mM in 0.05 M Tris-hydrochloride buffer of pH 7.5; 9.85 mCi/mmole). The first set was clotted by the addition of 40 μl of 25 mM calcium chloride; to the second set 0.04 unit of thrombin in 40 μl of 50 mM sodium chloride (in order to equalize ionic strengths) was added. Clotting (at 37°) ensued in both sets at approximately 3 min. The clots were squeezed out and removed at the times shown on the abscissa. They were washed individually with 0.15 M sodium chloride with mild shaking until no further radioactivity would pass into the supernatant and were then transferred into a 1-ml solution of chymotrypsin, containing 5 μg of crystalline (Worthington) enzyme in 0.1 M ammonium bicarbonate. Aliquots of 0.2-ml digests were taken for isotope counting. Values on the ordinate represent counts per minute for the total fibrin digests in Figure 7.

The electrophoretic experiment described in Figure 2 was carried out on thin-layer Brinkman cellulose sheet using a horizontal Savant high-voltage equipment, with 1% (v/v) pyridine–acetic acid buffer of pH 5.4. Following the application of a 2-μl sample, electrophoresis (*ca.* 40 V cm^{-1}) was performed (at about 10°) for a period of 2 hr. At the end of the run, the radioactive spots were located by cutting out sections of 0.5 × 2.5 cm (*i.e.*, 0.5 cm in the direction of migration and 2.5 cm in width), immersing them in 15 ml of Bray's scintillation fluid and counting.

Results

The reaction between monodansylcadaverine and benzyloxycarbonyl-L-glutaminylglycine offers one of the most sensitive tests (Lorand and Campbell, 1971) for assaying transglutaminase activity. The fluorescent γ-amide coupling product can be readily separated from the amine substrate and measured. The system is particularly useful in studying the inhibitory effects of nonfluorescent compounds on the enzyme and, as such, can be applied to isoniazid. On a molar basis (Figure 1), isoniazid was found to possess an inhibitory activity on the liver enzyme equal to that of histamine. The latter, with a measured $K_{M,app}$ of about 2×10^{-4} (Pincus and Waelsch, 1968), is a known amine donor substrate which is capable of competing in the reaction against monodansylcadaverine (Lorand and Campbell, 1971).

Though the experiment clearly proved that isoniazid was a potent inhibitor, it still remained to be shown in a stricter sense that the drug served as a substrate for the transglutaminase type of enzymes. In a transamidation reaction, isoniazid could conceivably act either as an acceptor (see arrow 1) or as a donor (see arrow 2) substrate. In the first case, the acti-



vated carbonyl group in the isoniazid molecule could serve as a target for a nucleophile (1); in the second, the amino group of the compound itself could lead in a nucleophilic attack (2).

Repeated trials, in the presence of transglutaminase, failed to demonstrate any reaction between [^{14}C]isoniazid and monodansylcadaverine. Since the latter was known to be a good amine donor substrate (Lorand and Campbell, 1971) it may be concluded that isoniazid cannot function as an acceptor in this enzyme system. Thus, if isoniazid was a transglutaminase substrate at all, it itself had to be of the donor type. The idea could be tested by analyzing an enzymatic reaction mixture comprising [^{14}C]isoniazid and benzyloxycarbonyl-L-glutaminylglycine, a known acceptor substrate for the liver enzyme. As seen in Figure 2, the enzymatic reaction gave rise

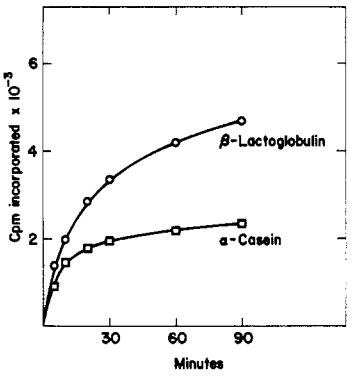


FIGURE 3: Transglutaminase-catalyzed incorporation of [^{14}C]isoniazid into β-lactoglobulin and α-casein. Concentrations of the latter proteins were 2.8 mg/ml; transglutaminase was used at an activity of 0.64 unit/ml. Isoniazid concentration was 0.7 mM (9.85 mCi/mmole).

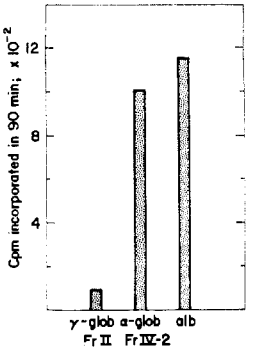
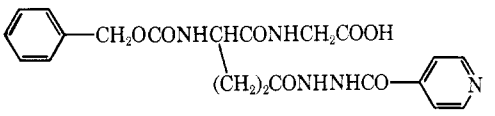


FIGURE 5: Various plasma proteins serving as acceptors for the transglutaminase-catalyzed incorporation of isoniazid. Enzyme activities were 1.4 units/ml; isoniazid was employed at concentrations of 1.4 mM (5 mCi/mmole). Concentrations of γ-globulin (fraction II) of α-globulin (fraction IV-2) and of albumin were 4 mg/ml. Reaction times were 90 min.

to a new product which accounted for about 88% of the isotope and migrated anodically at pH 5.4. Inasmuch as this compound could not be detected in any of the control experiments, it may be concluded that it represents the product between benzyloxycarbonyl-L-glutaminylglycine and isoniazid as shown in 1. As a result of the participation of labeled



(1)

isoniazid in this enzymatic coupling, the ^{14}C-carrying moiety would undergo a change in prototropic characteristics as shown by the electrophoretic findings.

The experiment described in Figure 2 may be taken as evidence that isoniazid is an amine donor, showing that this compound is active in the transglutaminase-catalyzed reaction by virtue of being a nucleophile. This, incidentally, is analogous to its participation in the acetylcoenzyme A dependent enzymatic acetyl transfer.

Having shown that isoniazid could actually serve as a substrate for transgultaminase, the incorporation of the drug into a variety of protein acceptors could be examined. The methodology described for other labeled amines such as histamine, putrescine, or monodansylcadaverine (Lorand and Campbell, 1971) could be used without modification.

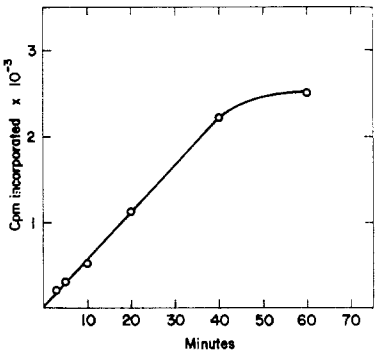


FIGURE 4: Transglutaminase-catalyzed incorporation of [^{14}C]isoniazid into human serum proteins. Enzyme activity was 0.84 unit/ml; isoniazid concentration as in Figure 2. The 75-μl reaction mixture contained 20 μl of human serum.

As such, the covalent coupling of isoniazid could be readily demonstrated to α-casein, β-lactoglobulin (Figure 3), or to human serum proteins (Figure 4). Uptake of the label indicated an average incorporation of approximately 1 mole of isoniazid into α-casein and 2 moles into β-lactoglobulin (at 90 min in Figure 3) for each mole of these proteins. Among the purified serum fractions surveyed (Figure 5), albumin and α-globulin (fraction IV-2) appeared to be most susceptible for enzymatic modification with isoniazid, whereas the γ-globulin preparation did not seem to incorporate significant amounts of this compound. These observations are in keeping with the recognized selectivity of transglutaminase toward protein acceptors (Clarke *et al.*, 1959; Lorand *et al.*, 1971).

The enzymatic labeling of proteins with isoniazid lends itself to a Michaelis type of analysis. A $K_{M,app}$ of about 1.3×10^{-3} was calculated for the transglutaminase catalyzed incorporation of isoniazid into β-lactoglobulin (Figure 6). A similar value was obtained for the incorporation of the drug with human serum serving as acceptor.

Circulating blood plasma is rather unique among tissues in that it does not possess any transglutaminase activity whatever. However, it contains a zymogen (called fibrin stabilizing factor or factor XIII) which, on treatment with thrombin (Lorand and Konishi, 1964; Lorand *et al.*, 1968b; Lorand and Gotoh, 1970) gives rise to a transamidase (called fibrinoligase; activated fibrin-stabilizing factor or activated factor XIII) playing an essential role in blood coagulation. Though specificity of the latter enzyme is appreciably different from transglutaminase (Myhrman *et al.*, 1970; Lorand *et al.*, 1971; Chung *et al.*, 1971), there are some significant similarities (Bruner-Lorand *et al.*, 1966). It will be recalled that this was the transamidase on which the effect of isoniazid was first noticed (Lorand and Jacobsen, 1967). Thus, it was of interest to examine whether this enzyme could bring about the covalent coupling of isoniazid to its natural fibrin substrate. As shown in Figure 7, there is a rapid uptake of isoniazid into fibrin, but only if clotting was carried out in the presence of calcium which permits the functioning of fibrinoligase (Lorand and Konishi, 1964). Clotting with thrombin in the absence of calcium produced virtually no coupling of the isotope to fibrin.

Considering the role of isoniazid as a transamidase substrate, discussed in relation to the experiment in Figure 2 and taking into account our present knowledge of the chemistry of fibrin cross-linking (Lorand *et al.*, 1968a; Lorand, 1970), it is almost certain that the observed inhibitory effect

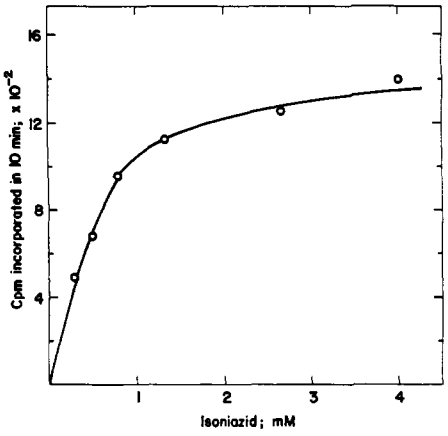


FIGURE 6: Dependence of velocity of transglutaminase-catalyzed incorporation of isoniazid into β-lactoglobulin on isoniazid concentration. The protein substrate was 5.3 mg/ml; enzyme activity 1.4 units/ml. Initial concentrations of [^{14}C]isoniazid (5 mCi/mmole) are shown on the abscissa.

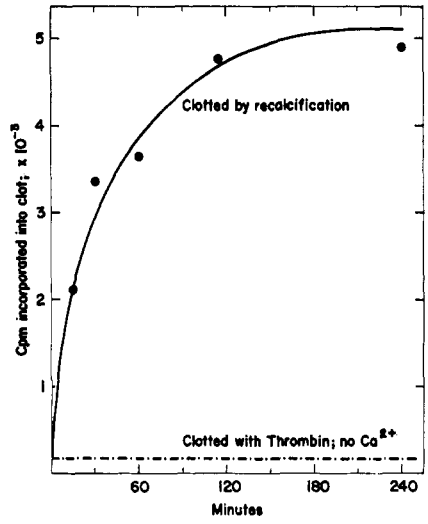


FIGURE 7: Uptake of isoniazid by fibrin during clotting of plasma with calcium. For explanation, see text.

of isoniazid on this process is due entirely to the blocking of the γ-glutamyl-acceptor sites in fibrin as is the case with the amine type of inhibitors in general. These compounds, as pseudodonors (or substitute), are known to compete with the ε-NH_2 lysine normal donors of fibrin (Lorand *et al.*, 1966) in the cross-linking reaction.

It should be mentioned that, in terms of inhibiting transglutaminase or fibrinoligase, another drug, hydralazine (1-hydrazinonaphthalazine), behaved exactly as isoniazid. Its effect, for example, could not be distinguished from that of isoniazid in the experiment shown in Figure 1. On account of its chemical structure, hydralazine can only be classified as a pseudodonor, *i.e.*, an amine type of inhibitor of transamidase-catalyzed reactions and would be expected to become enzymatically incorporated into proteins similarly to this group of substances (Lorand *et al.*, 1968a; Lorand, 1970).

Discussion

The finding that there are enzymes widely distributed in nature which can catalyze the covalent incorporation of isoniazid into proteins, merits consideration for two reasons.

First, it could conceivably have a bearing on the tuberculostatic effectiveness of the drug. If a critical step in the post translational assembly of proteins during the life cycle of the tubercle bacillus, *e.g.*, formation of a membrane of fibrous organelle, similarly to the enzymatic cross-linking of fibrin (Lorand *et al.*, 1962, 1968a; Lorand, 1970) depended on a transamidating mechanism, isoniazid would interfere as a substitute amine donor. It may be significant in this connection that isoniazid is already known to affect visibly the formation of the fertilization membranes of both *Lytechinus pictus* (E. Citkowitz, L. K. Campbell, and L. Lorand, unpublished results, 1971) and *Paracentrotus lividus* (F. Lallier, personal communication, 1971). However, it still remains to be seen whether there is a transglutaminase type of enzyme in the tubercle bacillus itself,[1] with a favorable enough $K_{M,app}$ for isoniazid to account for the specificity of the drug, and whether there exists in the bacterium an endogenous acceptor of the enzyme. In our work with sea urchins (*Lytechinus pictus* and *Arbacia punctulata*), high transglutaminase as well as endogeneous acceptor substrate activities were found (L. Lorand, L. K. Campbell, and G. Weismann, unpublished results, 1971).

Considered from another point of view, our finding may provide a possible explanation for the initiation of autoimmune response known to occur to a significant extent following the administration of isoniazid (and of related substances such as hydralazine; Alarcon-Segovia *et al.*, 1967; Perry *et al.*, 1970) either in man or in experimental animals.

If some tissue (*e.g.*, membrane) protein (P-$CONH_2$) is enzymatically modified by these drugs (H_2NR) extensively to yield an altered protein (P-CONHR), immunological tolerance toward the native protein might be broken. As such, antibody production against this normal tissue component could be stimulated long after the total elimination of the once altered protein.

The large number of proteins which can serve as acceptors for the transamidase-mediated incorporation of isoniazid, as described in this paper, is impressive. If modification by the drug induces antibody formation against a physiologically important site in the acceptor protein, the antibody would be expected to inhibit a recognizable biological function, while modification at other sites could go undetected. In this context, it may be of interest to mention that we have recently reported on two hemorrhagic disorders, both of apparently autoimmune origin in which it may be assumed that previous treatment with isoniazid played a role. One patient's serum contained IgG antibodies specific only against the fibrin stabilizing factor zymogen, thereby preventing its conversion to fibrinoligase (Lorand *et al.*, 1971); the other patient possessed similar type antibodies against fibrinogen and fibrin which did not seen to affect clotting but prevented the normal cross-linking of the fibrin substrate (Rosenberg *et al.*, 1971).

Since the modification of proteins by transglutaminase type of enzymes provides a plausible model for initiating the chain of autoimmunization in relation to drugs such as isoniazid, it will be pertinent to ask which tissue proteins are the best acceptor substrates for such compounds and which tissues are richest in transamidase activity. A preliminary survey (Clarke

[1] It may be mentioned that in two attempts we failed to demonstrate transglutaminase activity in homogenates of a nonvirulent, isoniazid-sensitive strain of *Mycobacterium tuberculosis* (H-37 Ra; kindly provided by Dr. G. Youmans). Neither [^{14}C]putrescine nor [^{14}C]isoniazid served as substrates for the homogenate even when either α-casein or β-lactoglobulin was added.

et al., 1959) which we confirmed, already shows that transglutaminase is a ubiquitous enzyme with particularly high activities occurring in liver, lung, spleen, kidney, muscle, and placenta. It is probably of the utmost physiological protective significance that normal blood does not contain any transamidase at all. In this tissue such activity arises only during the process of coagulation through the limited proteolysis of the fibrin stabilizing factor zymogen (factor XIII) by thrombin (Lorand and Konishi, 1964; Lorand *et al.*, 1968b).

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Adrenocorticotropin. Solid-Phase Synthesis of α^{1-19}-Adrenocorticotropic Hormone, Alanyl-α^{1-19}-adrenocorticotropic Hormone, and Prolyl-α^{1-19}-adrenocorticotropic Hormone and Their Adrenocorticotropic Activity*

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ABSTRACT: α^{1-19}-Adrenocorticotropic hormone nonadecapeptide, alanyl-α^{1-19}-adrenocorticotropic hormone eicosapeptide, and prolyl-α^{1-19}-adrenocorticotropic hormone eicosapeptide have been synthesized by the solid-phase method. A comparison of the *in vivo* steroidogenic potencies of the above peptides indicates that a one-residue extension of the peptide chain at the amino terminal lowers, but does not destroy, steroidogenic activity of α^{1-19}-adrenocorticotropic hormone.

Structure–activity studies on α-ACTH[1] and related peptides have, in part, been directed toward elucidating the importance of the free amino-terminal group for steroidogenic potency (Waller and Dixon, 1960; Lebovitz and Engel, 1963; Dixon, 1962). Synthetic peptides in which the amino-terminal serine has been replaced by proline, sarcosine, and

* From The Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received September 20, 1971.* This work was supported in part by grants from the National Institute of General Medical Sciences (GM-2907), the Geffen Foundation, and the Allen Foundation. K.-T. W. is a Fulbright Scholar for 1969–1971 and on leave from the Department of Chemistry, National Taiwan University, Taiwan. Paper XLI in the Adrenocorticotropin series. For paper XL, see Li and Hemmasi (1972).

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[1] All the asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration. Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(biphenyl)isopropyloxycarbonyl.