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Structural Relationships in the Interaction of Adrenocorticotropin with Plasma Proteins*

John E. Stouffer and Jen-Sie Hsu

ABSTRACT: Reverse thin film dialysis and gel filtration have been used to study the interaction and binding of adrenocorticotropin (ACTH) with proteins. Comparisons have been made with a tricosapeptide analog. A high degree of interaction is shown to occur between both of these peptides and proteins. Albumin is particularly effective in binding the hormone peptides, and this binding has been found to be concentration dependent.

Consistent and striking differences are observed between the bound and unbound forms of these peptides with regard to their hormonal behavior. From these results, and a knowledge of the portions of the peptide chains which are required for activity, some conclusions are drawn regarding the amino acid sequences which may be primarily involved in binding. The results indicate the probable major site of binding of the tricosapeptide to be at amino acid residues 15–18. It is also shown that amino acid residues 24–39 have an important effect on the hormone–protein interaction.

Interaction or binding of peptide hormones with proteins is difficult to study experimentally in terms of specific molecular structure. This is particularly true if these studies are to be carried out with the very small relative amounts of a peptide hormone which approach the physiological level. Measurements must then rely upon labeling techniques or some sensitive biological assay. Labeled peptides, pure enough for studies of this kind, are difficult to prepare and are not readily available. Biological assay procedures, while often tedious and frequently lacking in precision, nevertheless are capable of yielding information not obtainable by other methods.

The family of biologically active peptides related to

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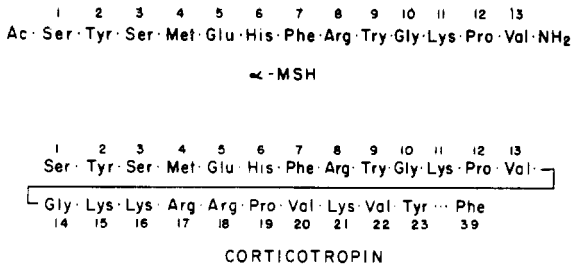

FIGURE 1: The amino acid sequences of α-melanotropin (α-MSH) and corticotropin (ACTH).

adrenocorticotropic hormone (ACTH)[1] and melanophore-stimulating hormone (MSH) offer a unique opportunity for a study of the relation of structure to protein binding. Not only may many of these peptide analogs be studied by two different biological assay systems, but much is already known about those amino acid sequences which are necessary to bring about the two responses. In these experiments, advantage has been taken of this to study the binding phenomenon in relation to the particular amino acid residues of the hormone peptide chain which may be contributing to the interaction. The reverse thin film dialysis technique previously used to study the binding of oxytocin with the pituitary protein neurophysin (Stouffer *et al.*, 1963) was utilized along with gel filtration in this work.

Thin film dialysis (Craig and Konigsberg, 1961; Craig *et al.*, 1964, 1965) has been shown to yield valuable information concerning the size and shape of large solute molecules. The large membrane surface area, relative to the volume inside the sac, allows a rapid rate of diffusion of even large peptides through the membrane. It had been shown (Stouffer *et al.*, 1963) that this apparatus could be used in the reverse manner, *e.g.*, by causing the peptide to diffuse into, instead of out of, the dialysis sac. If the solution inside the membrane contained a protein which was capable of binding the smaller peptide, it was observed that the peptide would be rapidly concentrated in this small volume compartment. In this manner it had been shown that, while the pituitary protein neurophysin has a very strong affinity for the peptide hormone oxytocin, the physiologically active synthetic analog, which lacks the N-terminal amino group, does not bind with this protein under the experimental conditions employed. Thus it became apparent that the primary amino group on the N-terminal cysteine residue of oxytocin must be involved in the protein–peptide interaction.

In the present work, studies were carried out with the natural adrenocorticotropic hormone and a highly potent analog, a tricosapeptide fragment (Hofmann *et al.*, 1961), corresponding with the first 23 amino acid residues of the N-terminal sequence of the natural peptide. Corticotropin and the tricosapeptide fragment both contain, in the N-terminal portion of their molecular structure, the entire sequence of α-melanotropin (α-MSH) as shown in Figure 1. Both of these larger peptides also have a high degree of melanophore-stimulating activity (Hofmann, 1962). Some unexpected differences between the bound and unbound forms of these peptides were disclosed when comparisons were made on the basis of both melanophore expanding and steroidogenic capabilities. The results, when considered in relation to what is known about the portions of the peptide chain which are necessary for the biological activities, permit some tentative conclusions to be drawn about those regions of the peptide chain which may be most involved in the binding with protein.

Experimental Procedure and Results

Materials. Human plasma albumin, human γ-globulin, and lyophilized whole human plasma were obtained from the Pentex Co., Kankakee, Ill. The synthetic tricosapeptide was a gift from Dr. Klaus Hofmann of the University of Pittsburgh. The natural porcine corticotropin was obtained through the purification of crude oxycellulose process concentrate, assaying 2–25 units/mg, which was a gift of the Armour Pharmaceutical Co. This material was purified for these studies by chromatography on a column of carboxymethylcellulose in a procedure similar to that used for the isolation of MSH and ACTH from extracts of the pituitary glands of other species (Lee *et al.*, 1960, 1961). The dialysis membranes were Visking cellophane dialysis tubing size 20/32. For these experiments the pore size of the membrane was not modified.

Biological Assays. These assays were carried out measuring both the *in vivo* steroidogenic and *in vitro* melanophore-expanding activities of these peptides. The intravenous, adrenocorticotropic activity was assayed against the U. S. Pharmacopoeia Reference Standard by measuring plasma-free corticosteroids using the fluorometric method (Silber *et al.*, 1958), modified by Guillemin *et al.* (1959). Hypophysectomy was replaced by Dexamethasone blockade. Dexamethasone 21-phosphate was a gift of Merck Sharp and Dohme Laboratory, West Point, Pa. Melanophore-stimulating activity was determined according to the procedure of Shizume *et al.* (1954) and Long and Guillemin (1961).

Carboxymethylcellulose Chromatography of Porcine ACTH. Crude porcine corticotropin was incubated at 80° for 1 hr in 0.1 M thioglycollic acid (Lee *et al.*, 1960, 1961). The mixture was adjusted to pH 5.5, and then diluted with 0.005 M ammonium acetate at pH 5.8 and applied to the column (10 × 180 mm) which had been equilibrated with the same buffer. The column was maintained at 4°, and elution carried out by the stepwise introduction of ammonium acetate solutions of increasing pH and molarity as shown in Figure 2. The fractions corresponding to the various peaks were lyophilized to remove ammonium acetate and peak IV was found to contain the bulk of the biological activity. The other numbered peaks gave material which

[1] Abbreviations used in this paper: ACTH, adrenocorticotropic hormone (corticotropin); MSH, melanophore-stimulating hormone (melanotropin).

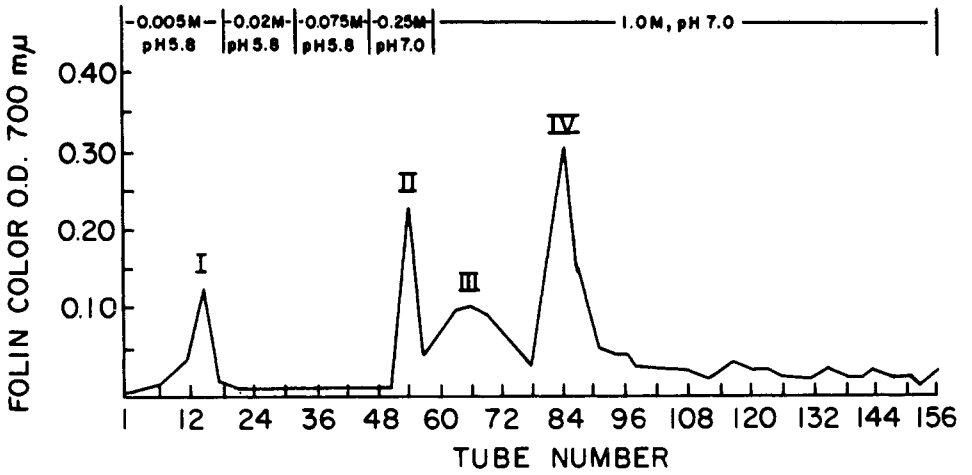

FIGURE 2: Chromatography of porcine ACTH on carboxymethyl cellulose. Ammonium acetate buffers of the composition shown across the top were employed in a stepwise manner as indicated, to elute the peptides. Fractions of 3 ml were collected. Peptide concentrations were determined by means of the Folin reaction (Lowry *et al.*, 1951).

had only low levels of steroidogenic activity and they were not investigated further. The small amounts of activity found in these other numbered peaks could have resulted from partial chemical or enzymatic degradation during the removal and storage of the pituitary glands and subsequent processing.

It is well known that porcine material isolated by the oxycellulose process contains several peptides with ACTH activity (Hofmann and Katsoyannis, 1963; Craig *et al.*, 1965). It is very probable that most of these are due to degradation of the natural material, although the existence of a true family of related peptides, as is apparently found in ovine preparations (Pickering *et al.*, 1963), cannot be ruled out. It is known, *e.g.*, that even very mild conditions, such as the presence of dilute ammonia, may cause the rapid conversion of glutamine in position 30 to a glutamic acid residue (Dixon and Stack-Dunne, 1955).

The purified porcine ACTH we thus obtained was found to have a potency, when assayed by the intravenous corticosteroidogenic assay procedure of about 40 units/mg. This is comparable to the potency found for other preparations of highly purified porcine ACTH when assayed according to this same procedure (H. S. Lipscomb, personal communication), or by the intravenous ascorbic acid depletion assay (Craig *et al.*, 1965).

Equilibrium Dialysis. The dialysis experiments were carried out in a cell similar to that previously described (Craig and Konigsberg, 1961). It had been observed (Stouffer and Lipscomb, 1963) that the tricosapeptide was very strongly adsorbed to glass surfaces in the neutral pH range and that natural corticotropin in a crude preparation behaved in a similar manner. It had also been shown that this glass surface binding was reversible and pH dependent. The presence of added protein also had an effect. A dialysis cell of similar dimensions but constructed of Teflon was therefore used in these experiments, and was found to permit complete recoveries of even trace quantities of these peptide hormones. The membrane was prepared for use by first soaking it in 10% phenol for 15 hr. It was then successively washed with distilled water, 0.1 N sodium hydroxide, 0.5% ethylenediaminetetraacetic acid disodium salt; and finally repeatedly with distilled water. Recovery of tricosapeptide and natural porcine corticotropin ranged from 85 to 110% in control runs, in the absence of protein, indicating no significant binding by the dialysis membrane or by the walls of the Teflon tubes.

A solution of protein, usually 50 mg, was dissolved in 1.0 ml of either 0.1 M sodium phosphate buffer, pH 7.4, or 0.1 M sodium acetate buffer, pH 5.0, and placed inside the membrane. The peptide hormone in buffer solution was placed in the tube outside the membrane. Dialysis was carried out at 25° and in a few instances at 4°. In most of the studies, the amount of tricosapeptide and that of porcine ACTH ranged from 0.5 to 3.0 μg. Preliminary experiments had shown that equilibrium could be reached within a period of 4 hr at 25° for the tricosapeptide and less than 12 hr at 25° for porcine ACTH. After equilibrium had been established, the volumes inside and outside the membrane were measured and appropriate dilutions made for bioassays. An aliquot of the outside solution was also tested by means of the Folin reaction (Lowry *et al.*, 1951) to make certain that the membrane had remained intact and no protein leakage had occurred.

The hormone distribution in many of the experiments was measured by means of the melanophore-stimulating assay. The reason for this will become more apparent later in this discussion. It was found that the presence of protein greatly affected the result of the steroidogenic bioassay but did not affect the melanophoretic assay to any appreciable extent.

The binding percentage of the hormone peptides with

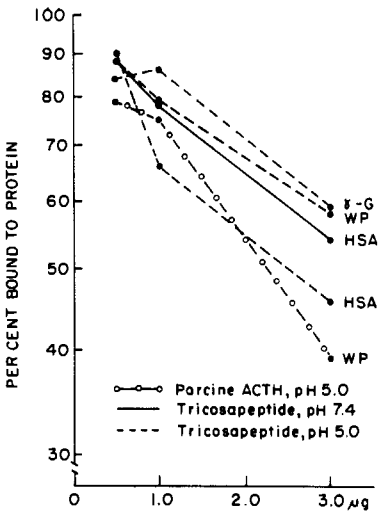

FIGURE 3: Reverse thin film dialysis of 1.0, 2.0, and 3.0 μg amounts of the tricosapeptide and porcine ACTH into solutions containing 50 mg of human serum albumin (HSA), 50 mg of human γ-globulin (γ-G) and 70 mg of lyophilized whole plasma (WP). Equilibration was obtained at 25° in either 0.1 N sodium acetate buffer, pH 5.0, or 0.1 M sodium phosphate buffer, pH 7.4.

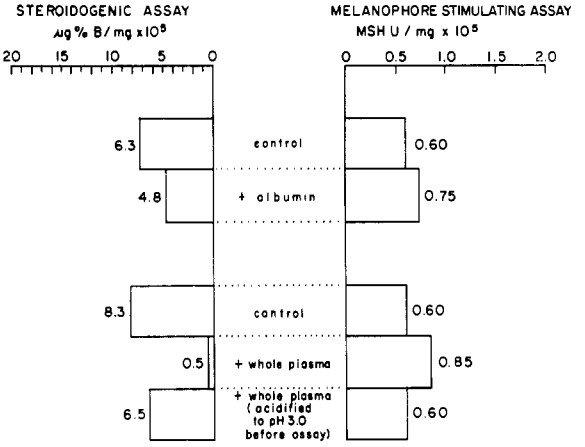

FIGURE 4: Effect of the presence of protein on the biological activities of the tricosapeptide after incubation at 4° for 16 hr in 0.1 M sodium phosphate buffer at pH 7.4. The tricosapeptide (1.0 μg) was mixed with a 1-ml solution containing 50 mg of human plasma albumin. A control containing no prote n was treated in an identical manner. A similar experiment with whole plasma is shown in the lower half of the figure. One of the three solutions was acidified with 0.1 N HCl to pH 3.0 before assay, as shown. The results of the steroidogenic assays are expressed in terms of μg of corticosterone (compound B)/100 ml of rat plasma.

the various proteins was calculated (Slaunwhite, 1960) according to the formula: % bound = $100[1 - (DVr/RVd)]$ where D and R are the amounts of hormone present inside and outside the dialysis membrane, respectively, and Vr and Vd are the corresponding volumes.

When the natural hormone or the synthetic tricosapeptide are permitted to equilibrate between a protein solution trapped inside the dialysis membrane and plain buffer solution on the outside, a very rapid and dramatic concentration of the peptide hormone occurs in the small volume side of the membrane containing the protein. At the beginning of the experiment all of the peptide hormone is in the outside, protein-free solution. However, the final concentration in the protein-containing solution may often be more than ten times that of the protein free solution outside the membrane. The results obtained are summarized in Figure 3. It is apparent that there is a strong binding affinity under these conditions, with a sharp dependence on the concentration of peptide. It is also apparent, that under these conditions, the natural porcine ACTH molecule is not bound as rigidly as is the tricosapeptide fragment. This is especially evident if one considers that equal weights of these peptides contain almost twice as many molecules of tricosapeptide as molecules of the natural hormone. It is probable that virtually all of these hormone molecules are protein bound in the living organism if one could extrapolate back to the very minute amounts which would normally be circulating in the blood stream.

Competitive Equilibrium Dialysis. On the basis of cold ethanol fractionation procedures it was considered (Bethune *et al.*, 1958) that ACTH might be transported in the blood in association with the globulin fraction, particularly the γ-globulins. A direct test of the relative binding capacities of γ-globulin and albumin under controlled *in vitro* conditions could be carried out utilizing the dialysis cell described previously. Two protein solutions separated by the membrane were allowed to compete with each other for a small amount of peptide hormone. It was considered that by such a direct comparison small differences between these two proteins in their binding capacities under these conditions might become more readily apparent. The results obtained for the competition between γ-globulin and albumin at pH 7.4 are shown in Table I. The hormone is seen to have migrated toward the albumin solution.

Effects of Protein Binding on Biological Potency. It had been observed by us in the early part of this work that the biological activities of these peptides, when measured by the steroidogenic assay, were profoundly affected by the presence of some proteins. A comparison was made of the effect of protein on the relative ability of the peptide hormones to evoke steroidogenic and melanophore stimulation. A series of separate experiments were then carried out in which a protein-containing solution was compared with a control solution maintained under identical conditions, and assayed at the same time, in the same group of animals. The tricosapeptide consistently gave lower steroidogenic responses in the

TABLE I: Competitive Dialysis between Human Plasma Albumin and γ-Globulin for Tricosapeptide.[a]

		Initial Hormone Concn(μg/ml)	Final Hormone Concn (μg/ml)
Inside Membrane:	10 mg/ml of γ-globulin	0.38	0.29
Outside Membrane:	10 mg/ml of albumin	0.38	0.45

[a] Sodium phosphate buffer (0.1 M) pH 7.4.

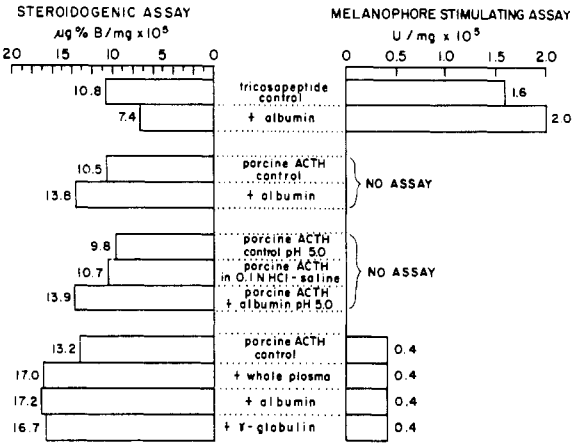

FIGURE 5: Effect of the presence of protein on the biological activities of porcine ACTH and the tricosapeptide after incubation at 4° for 16 hr in 0.1 N sodium acetate buffer at pH 5.0. The four separate experiments shown above were carried out in a manner similar to that described in Figure 4.

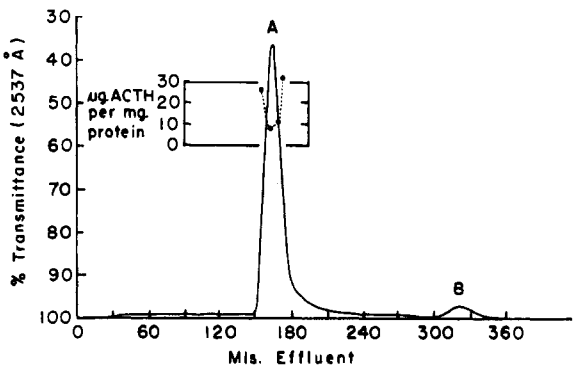

FIGURE 6: Gel filtration of a mixture of 15 mg of porcine ACTH with 50 mg of human plasma albumin in 0.1 N ammonium acetate buffer, pH 5.0, at 4°. Column: Sephadex G-25 (1.6 × 200 cm). Peak A: protein bound fraction; peak B; unbound hormone. The amounts of hormone bound to the albumin at several different regions of the protein peak were determined on the basis of the melanophore-stimulating assay because of the effect of protein on the steroidogenic assay, and are expressed as micrograms of ACTH per milligram of protein. The results are shown superimposed on the albumin peak, A. Protein concentration was determined by means of the Folin reaction (Lowry *et al.*, 1951).

presence of protein. The results of a typical experiment are illustrated in Figure 4. It is seen that binding with whole plasma appears to eliminate almost all the steroidogenic activity, while not affecting the MSH activity when compared to the control. At the same time and under the same conditions, incubation was carried out with another aliquot of the same solution of whole plasma proteins, but differing only in that it was acidified to pH 3.0 just before the assay. The potency of the hormone here was increased almost to the level of the control. Neither of these two solutions, or the control, differed significantly in their melanophoretic potencies. From these results it is clear that the observed relative loss of steroidogenic activity in the bound tricosapeptide could not be due to enzymatic cleavage of the peptide chain or to simple chemical alteration, *e.g.*, oxidation of the methionine residues. Neither could it be due to the binding of the hormone to glass, as had been observed earlier (Stouffer and Lipscomb, 1963), since the control solution remained highly potent. It is apparent that this effect is a result of the specific nature of the binding with protein.

The important fact that melanophoretic activity is not affected is an indication that binding may occur in the region of the molecule not involved in melanophoretic activity, *e.g.*, residues 14–23. That simple acidification permits a restoration of steroidogenic activity suggests that, at lower pH, the inactive bound complex is labilized, perhaps by the splitting of ionic or hydrogen bonds. Porcine ACTH does not lose steroidogenic activity when bound to protein as compared with a control run under identical conditions. This is in sharp contrast with the results obtained for the tricosapeptide under these same conditions as shown in Figure 5. It is apparent that in some way the amino acid residues from 24–39 in the natural hormone cause it to be bound to protein in a different manner.

Gel Filtration of Albumin–ACTH Complex. An additional experiment was carried out to determine the total specific binding capacity of human albumin for the natural hormone. To do this, a large amount of ACTH was mixed with the protein, and gel filtration on Sephadex G-25 was carried out in order to remove the excess

ACTH. The specific binding capacity was determined from the melanophoretic assay of various portions of the resultant albumin peak, and is shown in Figure 6. Even at a specific binding capacity of 30 μg of hormone/mg of protein there are still approximately two protein molecules for each bound peptide molecule. This could be a result of protein–protein interactions masking some sites on the protein molecules which would otherwise be available for binding with the peptide. Such a possibility is supported by the fact that even less peptide was bound per milligram of protein at the highest protein concentration than in tubes containing lower protein concentration representing either side of the peak.

Discussion

Bethune *et al.* (1958) suggested that ACTH in human plasma might be bound primarily to the γ-globulin and β-lipoprotein fractions. Corticotropin was added to human plasma which was then subjected to a cold ethanol fractionation procedure. ACTH activity was found associated mostly with fractions II and III (γ-globulin and β-lipoproteins) and lesser amounts in fraction IV (α- and β-globulins). In contrast with this, it is apparent from our results that albumin has a very high binding capacity, and particularly if one considers the overwhelming relative amount of this protein present in the blood, it would seem reasonable that a large part, if not virtually all, of the circulating hormone is bound to albumin. The ultracentrifuge studies of Brown *et al.* (1955) on the behavior of ACTH have also demonstrated binding in the presence of albumin.

It has been shown (Hofmann *et al.*, 1963; Dedman *et al.*, 1961) that the methionine sulfur, while not essential for activity is, in the sulfoxide form, capable of interfering with the formation of an active hormone–receptor complex. An inactivation–activation phenomenon, based on the reversible oxidation of the methionine residue, does not appear to be involved in the dramatic changes occurring when the tricosapeptide is bound to protein. Melanophore-stimulating activity was not impaired, as it would have been had the methionine residue been oxidized (Lo *et al.*, 1961), and it is difficult to conceive of simple acidification leading to the reduction of a methionine sulfoxide residue.

The implications of the experimental results obtained here may become clearer, if one again considers the structure of these peptides (Figure 1). That portion of the tricosapeptide which includes residues 14–23 appears to be associated with the strong interaction with protein. In the sequence from 14 to 23, the only amino acid residues which contain side-chain functional groups other than hydrocarbon, and which might be expected to contribute significantly to binding with protein, are those strongly basic residues from 15 to 18. These residues are also those which are necessary in order to obtain a high level of adrenocorticotropic activity.

The enhancement of the steroidogenic activity of the natural ACTH molecule is in sharp contrast with the consistently lowered potency of the 23 amino acid fragment, when each of them are mixed with protein. The apparent lower degree of conformational stability of the tricosapeptide relative to that of the natural hormone as observed by Craig *et al.* (1965) could very well be a contributing factor to this phenomenon.

These differences between the synthetic tricosapeptide and natural porcine ACTH clearly demonstrate that the amino acid residues from positions 24 to 39 have an important effect on the interaction between the natural peptide hormone and protein molecules.

While these studies have emphasized the interaction of this hormone with protein, it must not be overlooked that at some stage in the mechanism of action of this hormone, a combination of the corticotropin peptide directly with a ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecule might also be occurring. A suggestion of this kind has been made (Schwyzer, 1964) in connection with a possible explanation of the effects of ACTH on protein synthesis. This has been based primarily on the observation of increased RNA synthesis in adrenal tissue upon ACTH stimulation (Bransome and Reddy, 1961; Scriba and Reddy, 1965) and the effect of puromycin inhibition on ACTH stimulation of adrenal slices (Ferguson, 1962). Whether these are primary results of ACTH action or due to secondary effects is not known, and a certain amount of caution is warranted before conclusions are drawn (Korner, 1965). Some structural features of this molecule, however, do lend some support to such a speculation. A concentration of basic amino acid residues, as those in positions 15–18, is seldom observed outside the nuclear proteins, and it is perhaps also more than a coincidence that the same N-terminal Ser-Tyr-Ser sequence, containing three side-chain hydroxyl groups in a row, occurs as the N-terminal sequence of at least one virus protein (Tsugita *et al.*, 1960).

Acknowledgment

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