upon admixture with an authentic sample, $[\alpha]^{25}D$ −72.4°, ultraviolet$_{max}$ 242, 250, 260 mμ (log ε 4.44, 4.50, 4.31), brown coloration with tetranitromethane.

Anal. Calcd. for $C_{30}H_{48}$: C, 88.16; H, 11.84. Found: C, 88.02; H, 11.91.

Friedelan-3α-ol Chloroacetate.—A solution of 600 mg. of friedelan-3α-ol in 50 ml. of lepidine was cooled until the lepidine just started to crystallize. To this solution was added 1.5 ml. of chloroacetyl chloride dropwise with swirling. The mixture turned dark brown and a precipitate appeared immediately. The mixture was left without further cooling for 0.5 hr., diluted with water and extracted with ether. The ether extract was washed with dilute hydrochloric acid, 1% aqueous sodium hydroxide and water. Charcoal treatment of the ether layer gave a light brown solution which was concentrated to a small volume, 10 ml. of ethanol added and the remaining ether was removed. The small plates which separated were filtered, dried and dissolved in benzene. The benzene solution was filtered through a column of alumina and evaporated to dryness. Crystallization of the residue from methylene chloride–ethanol gave 340 mg. of colorless plates, m.p. 280° dec. Two further crystallizations raised the melting point to 283° dec.; infrared$_{max}$ (carbon disulfide) 1760, 1732 cm.$^{-1}$. This material was satisfactory for X-ray single crystal analysis.

Anal. Calcd. for $C_{32}H_{53}O_2Cl$: C, 76.07; H, 10.58. Found: C, 76.34; H, 10.16.

Friedelan-3α-ol Bromoacetate.—A solution of 750 mg. of friedelan-3α-ol, 2 ml. of bromoacetyl bromide and 2 ml. of pinene in 30 ml. of methylene chloride was heated to reflux for 20 hr. Heptane was added and the methylene chloride was distilled off. Upon cooling the heptane solution deposited 680 mg. of bromoacetate which was purified by copious washing with hexane, passage through a short column of alumina in benzene solution and recrystallization from benzene; 650 mg., m.p. 279°. Recrystallization from *n*-heptane–methylene chloride afforded analytically pure material, m.p. 279°. Samples for X-ray analysis were prepared by recrystallization from benzene.

Anal. Calcd. for $C_{32}H_{53}O_2Br$: C, 69.92; H, 9.72; Br, 14.54. Found: C, 69.99; H, 9.57; Br, 14.41.

URBANA, ILLINOIS

[CONTRIBUTION FROM EXPERIMENTAL THERAPEUTICS, MEDICINAL CHEMICAL, AND VIRAL AND RICKETTSIAL SECTIONS, RESEARCH DIVISION, AMERICAN CYANAMID CO.]

Studies with Corticotropin. I. Isolation, Purification and Properties of β-Corticotropin

BY R. G. SHEPHERD, K. S. HOWARD, P. H. BELL, A. R. CACCIOLA, R. G. CHILD, M. C. DAVIES, J. P. ENGLISH, B. M. FINN, J. H. MEISENHELDER, A. W. MOYER AND J. VAN DER SCHEER

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Seven active components have been separated by countercurrent distribution of oxycellulose hog corticotropin. The homogenous principal component has been characterized by end group and quantitative amino acid analysis. Two types of intermedin activity present in crude corticotropin preparations were separated.

The isolation, purification and characterization of β-corticotropin have been briefly reported[1] earlier. The results of this investigation are given in detail here and in the following papers.[2,3]

The physiological importance of the hormones of the anterior pituitary gland has been the subject of many papers in the last two decades. It was recognized that certain of these materials were involved in the formation of steroids by the adrenal gland. However, the announcement that ACTH was effective in rheumatoid arthritis[4] stimulated research workers to the efforts which have resulted in the appearance of several hundred reports in the literature of the last six to eight years. Based on our current knowledge, the bulk of this work was carried out using very impure material.

The existence of more than one type of pituitary hormone activity which affected the adrenal cortex was first suggested by Reiss, *et al.*[5] It was later proposed[6] that the two suspected factors be designated the *adrenal weight factor* and the *ascorbic acid factor*. However, this concept has been questioned,[7a] since the apparent separation of the two factors could not be confirmed when the method of administration of the sample was varied. The present work was not designed to resolve this question. Rather, we were interested in preparing a pure, clinically active hormone.

The term "corticotropin activity" as used here refers to response in the adrenal ascorbic acid depletion assay of Sayers, *et al.*[8] We consider the hormonal activity measured by this assay to be significant, since the clinical activity[9] in rheumatoid arthritis appeared to be proportional to the Sayers assay response for "clinical" ACTH, for β-corticotropin and its pepsin degradation product[2] P4.

Early work by Li, *et al.*,[10] and Sayers, *et al.*,[11] led to the conclusion that ACTH isolated from either hog or sheep pituitaries by acid–acetone extraction[12] was a pure protein of molecular weight about 20,000. The peptide character of its active principle was demonstrated by its solubility properties and its inactivation by proteolytic enzymes such as trypsin,[13,14] chymotrypsin,[14] pepsin,[14] and carboxypeptidase.[13] The active principle was sepa-

(1) P. H. Bell, *et al.*, THIS JOURNAL, **76**, 5565 (1954); K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies and P. H. Bell, *ibid.*, **77**, 3419 (1955).

(2) II. P. H. Bell, *et al.*, *ibid.*, **78**, 5067 (1956).

(3) III, R. G. Shepherd, *et al.*, *ibid.*, **78**, 5067 (1956).

(4) P. S. Hench, E. C. Kendall, C. H. Slocumb and H. F. Polley, *Proc. Staff Meetings Mayo Clinic*, **24**, 181 (1949).

(5) M. Reiss, J. Balint, F. Oestreicher and V. Aronson, *Endokrinologie*, **18**, 1 (1936).

(6) F. G. Young, *Lancet*, **260**, 1211 (1951); F. G. Young and M. Stack-Dunne, *Brit. Med. J.*, **1**, 1386 (1951).

(7) (a) E. B. Astwood, M. S. Raben and R. W. Payne, "Recent Progress in Hormone Research," Vol. VII, Academic Press, New York, N. Y., 1952, pp. 4–8; (b) *ibid.*, pp. 40–42; (c) *ibid.*, pp. 28–30.

(8) M. A. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1948). Intravenous administration was used.

(9) E. B. Astwood, New England Center Hospital, Boston 11, Mass., personal communication.

(10) C. H. Li, H. M. Evans and M. E. Simpson, *J. Biol. Chem.*, **149**, 413 (1943).

(11) G. Sayers, A. White and C. N. H. Long, *ibid.*, **149**, 425 (1943).

(12) W. R. Lyons, *Proc. Soc. Expil. Biol. Med.*, **35**, 645 (1937).

(13) J. B. Lesh, J. D. Fisher, I. M. Bunding, J. J. Koosis, L. J. Walaszek, W. F. White and E. E. Hays, *Science*, **112**, 43 (1950). Based on our current knowledge, the apparent inactivation by carboxypeptidase was probably due to contamination by trypsin and chymotrypsin.

(14) C. H. Li and K. O. Pedersen, *Arkiv för Kemi*, **1**, 533 (1950).

rated from the "pure" 20,000 molecular weight protein by Astwood, *et al.*,[7] by a simple process. Thus, the 20,000 molecular weight protein would represent either a crude form of the hormone or a definite complex of an inactive protein with a smaller active component. The starting material for our studies had been purified by the glacial acetic acid procedure of Payne, *et al.*,[15] which was applied to acetone-dried hog anterior pituitary powder. This product[16] contained 1–2 U.S.P. units/mg. and was designated "clinical" ACTH. It had been shown by Payne, *et al.*,[15] that such "clinical" grade material could be further purified by cellulose adsorption columns which were superior to their partition, adsorption or ion exchange columns.[7c] In the early stages of our work, the cellulose adsorption process was varied extensively without improving the procedure recommended by these authors. Purification of the cellulose column material was attempted by many conventional procedures, and although its inhomogeneity was apparent, no satisfactory fractionation was produced.

With the report of Astwood, *et al.*,[17] of the 40-fold concentration of the activity of "clinical" ACTH[15] using adsorption on oxycellulose, this elegant purification technique was adopted here and their results were confirmed. 80% of the activity[18] was retained in a fraction representing 2% of the total weight, a separation which had not been approached by a variety of other methods attempted in these laboratories. The material was consistently active at >80 U.S.P. corticotropin units/mg. and readsorption of the activity on oxycellulose at varying ratios of charge to adsorbant did not increase the potency further. Such preparations also contained essentially all of the intermedin activity which was present in "clinical" ACTH. Both kinds of activity were followed through purification procedures in order to determine whether these activities reside in the same or in different molecules. The activities are discussed below and later[3] in relation to homogeneity and to the peptide structure.

In attempting further fractionation of oxycellulose–purified corticotropin (OC–ACTH), a great many methods of peptide and protein purification were investigated, including *p*H precipitation; precipitation with salts; insoluble salt formation (picrates, flavianates, picrolonates, sulfonates); dialysis; ultracentrifugation; electrophoresis; paper strip chromatography; Hyflo and powdered glass partition columns; Solka Floc and filter paper pulp adsorption columns; Amberlite IRC-50, Dowex-50 and Nalco X-219 ion exchange columns; and countercurrent distribution. Of these methods, countercurrent distribution proved to be the only one which demonstrated really effective resolution of the OC–ACTH mixture combined with good recovery of activity. Fractionation by countercurrent distribution was therefore extensively investigated.

Initially, a survey of some 250 solvent systems was made using a radio-iodine derivative of OC–ACTH. Radiation count analysis made rapid investigation possible and required only ultramicro quantities of material. The systems of promise were then examined more carefully with the unsubstituted hormone.

Four types of possibly useful systems evolved from the study: (1) phenols *vs.* water, (2) lutidines *vs.* water, (3) butanols *vs.* aqueous acids (trichloroacetic, trifluoroacetic, acetic, in decreasing order of distribution coefficient), and (4) butanols *vs.* aqueous salt solutions. The first two types, however, yielded emulsions which made them impractical for use in an automatic distribution apparatus. The coefficient in the type 4 system increased with increasing salt concentration and the decreased solubility caused by the salt was overcome by adding an acid (preferably acetic). 1-Butanol was vastly superior to 2-butanol for resolution[2] in the type 3 systems, and was less susceptible to peroxide formation. No inactivation of the dissolved corticotropin was observed.

The distributions were followed by analysis for nitrogen or for protein, and in all the key experiments biological assay was used concurrently with other analyses.

The separation of OC–ACTH which is provided by 1-butanol *vs.* 0.5% trichloroacetic acid (TCA) is shown in Fig. 1. Each peak had both corticotropin and intermedin activity, but in radically different proportions. The fast component, T(6–10), contained 80% of the weight and 90% of the corticotropin activity of the charge, with 20% of the weight and 10% of this activity remaining in the slow fraction, B(1–5). Distribution of the B(1–5) fraction in 2% TCA *vs.* 1-butanol led to its further fractionation, as shown in Fig. 2. The δ_1-fraction accounted for about 50% of the weight of B(1–5), essentially all of its corticotropin activity and about 90% of the original intermedin activity of OC–ACTH. The remaining 10% of intermedin activity, which was qualitatively and quantitatively different from that of the δ_1-fraction, was present in the T(6–10) peak of Fig. 1. The intermedin activity of this peak was unchanged after redistribution for 200 transfers and persisted in all samples having corticotropin activity.[3] Attempts to find some separation of this type of intermedin activity from corticotropin were unsuccessful, although the products from all purification methods were examined. The persistence of a constant intermedin-to-corticotropin activity ratio in these samples makes it highly unlikely that the intermedin activity is due to an impurity.

Attempts to fractionate T(6–10) by countercurrent distribution using 1-butanol with lower TCA concentrations or with toluene added were unsuccessful. However, a type 4 system, 3.5% sodium chloride in 6.0% acetic acid *vs.* 1-butanol,

(15) R. W. Payne, M. S. Raben and E. B. Astwood, *J. Biol. Chem.*, **187**, 719 (1950).

(16) This starting material was generously supplied by Dr. David Klein, Wilson and Co., Inc., Chicago, Ill.

(17) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1951).

(18) The actual units of corticotropin activity recovered by this method amount to somewhat less than that originally present, as measured by the Sayers assay. The assay has been shown to be sensitive to a "foreign protein effect" (*cf.* ref. 19).

(19) R. A. Brown, A. W. Moyer, M. C. Davies and H. R. Cox, *Arch. Biochem. Biophys.*, **58**, 68 (1955).

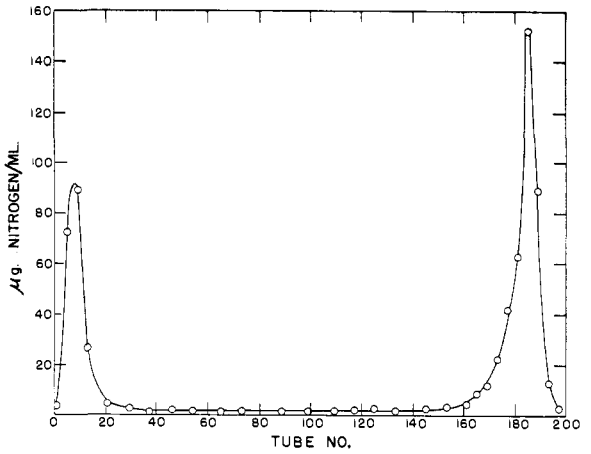

Fig. 1.—Distribution of OC–ACTH in 0.5% TCA *vs.* 1-butanol after 199 transfers (413 mg. charged in 2 tubes).

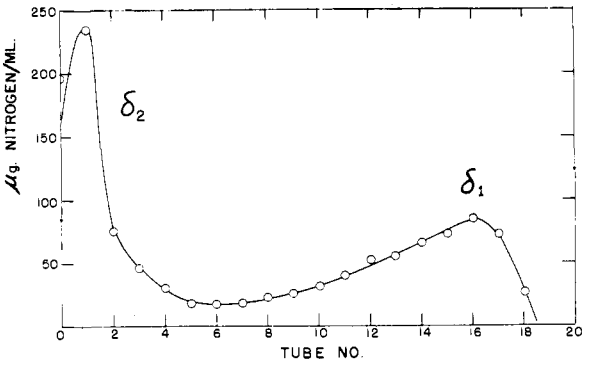

Fig. 2.—Distribution of B(1–5) in 2.0% TCA *vs.* 1-butanol after 19 transfers (504 mg. charged in 1 tube).

separated the material into at least four components (Fig. 3). The percentages of the three pools of components were 11% α, 66% β and 23% γ. The reality of the initial separation into the α-, β-, γ_1- and γ_2-components of Fig. 3 was demonstrated by redistribution of each in the same solvent system (Figs. 4 and 5). In addition, the α- and γ-mixtures were each shown to contain three components. The δ_1-component was shown to be different from the γ-material by a similar redistribution. The reproducibility of the original distribution coefficients (K), using the isolated components, showed there had been no interaction of these proteins. No appreciable variation of K with concentration was noted. The presence of a small amount of material (α_4) preceding the β-peak (Fig. 4) was demonstrated to be due to its formation from β during the first distribution.

Application of the same countercurrent distribution to OC–ACTH itself resulted in similar fractionation of the components: 12% α, 49% β, 19% γ and 20% δ. The unremoved B(1–5) material remained at the origin, as expected from Fig. 5. It was found, however, that the δ-fraction was very difficult to recover from the salt system and, consequently, TCA distribution is desirable for isolation of δ from OC–ACTH. All these fractions, excepting the δ, showed equal biological activity at about 100 U.S.P. units/mg. by the Sayers assay.

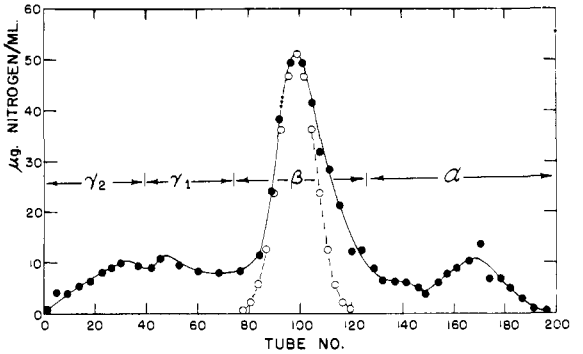

Fig. 3.—Distribution of T(6–10) in 3.5% NaCl in 6.0% HOAc *vs.* 1-butanol after 207 transfers (30.4 mg. charged in 3 tubes): O- - -O, theoretical curve.

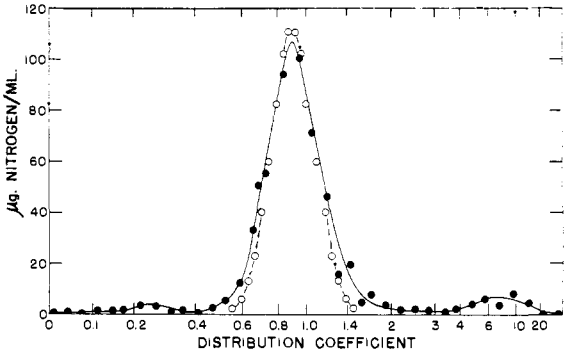

Fig. 4.—Redistribution of β-corticotropin in 3.5% NaCl in 6.0% HOAc *vs.* 1-butanol after 138 transfers (357 mg. charged in 4 tubes): O- - -O, theoretical curve.

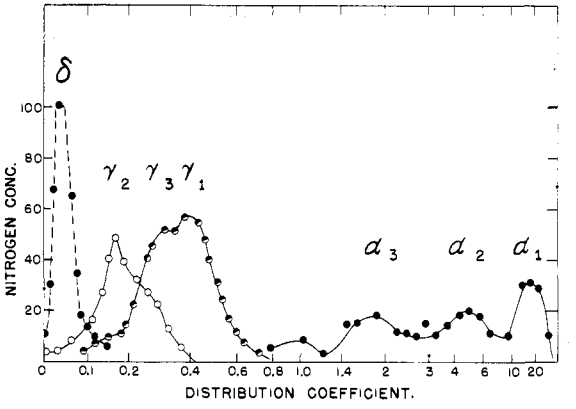

Fig. 5.—Redistributions of other corticotropins in 3.5% NaCl in 6.0% HOAc *vs.* 1-butanol: α-pool, n = 200, ●——●; γ_1-pool, n = 411, ◒——◒; γ_2-pool, n = 393, O——O; α-pool, n = 138, ●- - -●.

The β-fraction was selected for further characterization because it represented the largest single active component (*ca.* 50%) of OC–ACTH and it was digested by pepsin to only three active products.[2] The α- and γ-fractions, on the other hand, each gave six to eight products on pepsin digestion, and the T(6–10) material and OC–ACTH each gave at least seven groups of products.

Larger quantities of the β-corticotropin were prepared directly from OC–ACTH by countercurrent distribution in the salt system. The α- and

γ-components were removed from the distribution at intervals, and the β-component was extracted for a total of 921 transfers. At this time the distribution curve was essentially theoretical in shape and distribution coefficients determined by nitrogen analysis were constant for selected tubes across the peak. This β-corticotropin was considered to be homogeneous and was isolated and studied for homogeneity by other means. The material was active at about 100 U.S.P. corticotropin units/mg., with an occasional assay as high as 300 units/mg., and contained some intermedin activity[3] as well.

In contrast to the T(6–10) material, β-corticotropin appeared to be homogeneous in the ultracentrifuge, where it was studied at μ 0.10 (0.05 M potassium chloride, 0.05 M hydrochloric acid).[20] Treatment of the data by the method of Archibald[21] gave a value of 4720 for the molecular weight of the free base of the hormone, after correcting for charge effects.[20] Amino acid analysis by the Dowex-50 method of Moore and Stein[22] gave the following empirical formula

$$Ala_3Arg_3Asp_2Glu_5Gly_3His_1Leu_2Lys_4\text{-}Met_1Phe_3Pro_4Ser_2Try_1Tyr_2Val_3(NH_3)_1$$

The minimum formula weight of 4567 derived from this analysis is in good agreement with the molecular weight obtained from the ultracentrifuge data. Analysis of the acetate salt for carbon, hydrogen, nitrogen and water gave values which agreed with the above empirical formula, with 32 moles of water per mole of peptide base. This hydrate was stable to drying at 0.01 mm. at room temperature, but the water could be removed by drying at 10^{-6} mm. at 100°. The hydrochloride salt formed a similar hydrate which was stable even to drying under the latter conditions. Smaller synthetic peptides such as arginylleucine,[23a] leucylalanylvalylphenylalanylglycylproline[23b] and tryptophanyllysylleucylalanylvalylphenylalanylglycylproline[23b] also form stable hydrates. The C/N ratio of the hydrochloride salt was identical with the value of 210/56 calculated from the amino acid formula.

The homogeneity of β-corticotropin was further demonstrated by the single N-terminal amino acid and the single C-terminal amino acid sequence. The dinitrophenyl end-group procedure of Sanger[24] indicated that only serine was N-terminal. Because of the recognized alkali-lability of β (*vide infra*), it was desirable to demonstrate the presence of an N-terminus by methods employing other than the usual alkaline conditions. The methods used were reductive methylation and periodate oxidation, each at pH 5. The results of both these procedures substantiate the conclusion that one mole of serine is N-terminal in β-corticotropin prior to any exposure to alkali. Carboxypeptidase digestion of the hormone showed a single C-terminal sequence of . . .Leu–Glu–Phe·OH.

All these data seemed sufficient to justify the conclusion that β-corticotropin was essentially homogeneous and was suitable for studies designed to produce a smaller active molecule[2] and for determination of structure.[3]

The stability of the biological activity of β-corticotropin was investigated in some detail, using both enzymatic and chemical degradation. The activity was entirely stable to pepsin digestion[2] for 1 hour at enzyme:substrate ratios as high as 1:38 and to carboxypeptidase digestion for at least 40–60 hours. With trypsin and chymotrypsin, however, total inactivation was observed[3] within 30 minutes at enzyme:substrate ratios of 1:10. Activity persisted after 16 hours boiling in 0.1 N hydrochloric acid and after 64 hours exposure to 0.1 N hydrochloric acid at 25°.

The molecule was considerably more sensitive to alkali treatment. Boiling for 20 minutes in 0.1 N sodium hydroxide produced a marked decrease of the ACTH response and a striking potentiation of the intermedin response.[3] The salt distribution curve of such alkali-treated β gave evidence of several new components, and the most active had the same distribution coefficient as the δ-material in the 0.5% TCA system. Boiling for 25 minutes in 10 N ammonium hydroxide or 15% triethylamine was not sufficient to accomplish this alkaline conversion. End-group studies by a modification[3] of the phenyl isothiocyanate reaction showed that some serine, seryltyrosine and seryltyrosylserine had been removed from the N-terminus by the alkaline boiling of β-corticotropin.

Exposure of the molecule to pH 9 sodium bicarbonate (0.1 M) at 25° for 18–22 hours resulted in deamidation, without alteration of activity. The use, in purification procedures, of ion exchange columns such as Amberlite IRC-50 (XE 64) with alkaline eluents, as reported by White and co-workers,[25] may be sufficient to effect deamidation. Therefore it is possible that Corticotropin-A[26] isolated in this way is the deamidated form of the natural hormone β-corticoptropin. Li, *et al.*,[27] have similarly removed two of the four amide groups of the natural sheep corticotropin by alkaline treatment in the course of preparation of "α-corticotropin."[28]

The interrelationship of the active substances other than β was considered. Countercurrent distribution of β which had been incubated with glacial acetic acid at 70° for 4 hours showed it to be unchanged with no evidence of γ-type material and little, if any, conversion to α-type material. This result shows that the glacial acetic acid treatment used in the preparation of "clinical" ACTH did not produce these fractions from β.

The chemical relationship between these active α-, β- and γ-materials has not been completely clarified, but their differences appear to be minor. Paper strip studies of the isolated α-, γ- and δ-fractions revealed no qualitative difference in amino

(20) Studies with Corticotropin. IV: R. A. Brown, M. Davies, M. Englert and H. R. Cox, THIS JOURNAL, **78**, 5077 (1956).

(21) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947).

(22) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(23) (a) G. W. Anderson, THIS JOURNAL, **75**, 6081 (1953); (b) J. R. Vaughan, Jr., and J. A. Eichler, *ibid.*, **76**, 2474 (1954).

(24) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(25) W. F. White and W. L. Fierce, THIS JOURNAL, **75**, 245 (1953).

(26) W. F. White, *ibid.*, **75**, 503 (1953).

(27) C. H. Li, I. I. Geschwind, J. S. Dixon, A. L. Levy and J. I. Harris, *J. Biol. Chem.*, **213**, 171 (1955).

(28) Li, *et al.* (*cf.* ref. 27), have used the Greek letters α, α_2 and β in an entirely different sense for the products derived from sheep corticotropin.

acid content, either among themselves, or from β. The T(6–10) material, about half of which was α- and γ-material, showed only slight quantitative differences in amino acid content from β. The following studies of T(6–10) did not distinguish it from β. By reaction of T(6–10) with dinitrofluorobenzene it was found that the lysine Σ-amino groups and the tyrosine hydroxyl groups were all free. Its average molecular weight, based on ultracentrifuge studies, was 3900–4500. Carboxypeptidase digestion liberated one-third of its total phenylalanine. Thus, the only observed difference between all these corticotropins was in the products of their pepsin digestion. All the above comparisons suggest that the α-, β- and γ-fractions are very similar in structure.

Some of the α-material may be related to β by simple esterification. Prolonged distribution of β in the salt system showed the formation of a small amount (< 10%) of a material designated α_4 which, on redistribution, had a higher coefficient than that calculated from its position in the original distribution. In addition, isolated β-fractions which had been completely freed of α-material consistently revealed a small amount of this α-component on redistribution (Fig. 4). Later work suggested that the material had low reactivity to carboxypeptidase, and the theory that α_4 might be esterified β was postulated. Substantiating this theory, a peptide fragment, T2, which represents the esterified C-terminal sequence of β is formed[3] in an amount proportional to the α_4-contaminant. No attempt was made to prepare esterified β for comparison with α_4 because of the expected complexity of the esterification of a molecule containing seven free carboxyl groups. Various alkoxyl determinations were not useful because of trace contamination by alkylamines introduced by the ion exchange resins used in the recovery process.

The structures of the α-, γ- and δ-fractions were not further investigated. β-Corticotropin was degraded to smaller active molecules as reported in the succeeding paper.[2] The complete structures of these products and of β were then elucidated,[3] principally by enzymatic degradation. The countercurrent purification and enzymatic degradation which were used for the study of β would also be expected to be successful with the α-, γ- and δ materials because of the evidently close similarity of the fractions.

Experimental

Cellulose Studies.—The findings of Payne, Raben and Astwood[15] were confirmed. In addition, further elution with 0.1 *N* hydrochloric acid in 50% ethanolic solution produced a second active band which was differentiated from the first band by paper chromatography. The first band contained two-thirds of the total activity recovered (25–30%). This material contained traces of threonine, cysteine and ornithine in addition to the amino acids of β-corticotropin.

This first band on countercurrent distribution in 1% trifluoroacetic acid *vs.* 2-butanol gave three peaks (K = 0.3, 1.7 and 8.8), the latter two of which were active. Paper chromatography of hydrolysates showed no qualitative nor large quantitative difference between these two active peaks. Only one of these (K 2.0) was present in OC–ACTH and the other has not been further investigated.

Oxycellulose Purification.—The Astwood[17] oxycellulose purification procedure was repeated, using 11% oxidized cellulose (Tennessee Eastman) washed with water, 1.0 *N* hydrochloric acid, water and 0.1 *N* acetic acid. The "clinical" ACTH (0.025 g./ml.) was adsorbed on the oxycellulose (charge:adsorbant ratio 12.5:1) from 0.1 *N* acetic acid, leaving less than 5% of the activity unadsorbed. Elution with 0.1 *N* hydrochloric acid was carried out until the 2750 Å. absorption reached a constant low value. (Subsequent elution with 1.0 *N* hydrochloric acid or 0.1 *N* hydrochloric acid in 50% ethanolic solution produced little or no additional material.) The pooled eluates were treated with freshly washed Amberlite IRA-400 acetate to *p*H 3.5, the resin was removed by centrifugation and the supernate lyophilized. Total weight yield of this white acetate was 1.9, 1.9 and 2.1 in three runs, and activity recovery was >80%. Retreatment of the mother liquors with twice as much fresh oxycellulose produced a further small amount of material of lower biological activity.

Distribution Studies.—An extensive survey of solvent systems was carried out on radio-iodinated corticotropin after preliminary investigation in a few acid, neutral and alkaline systems showed that its properties paralleled those of corticotropin. This survey was made by single tube equilibrations followed by radiation count analysis on dried aliquots of each phase. The most suitable systems (about 25) were then applied to the unsubstituted hormone using 10- and 25-tube distributions. In view of the paucity of information on distribution properties of proteins, some general conclusions are given below and a portion of this work is given in Tables I and II. Several of these systems showed promise for resolution of the T(6–10) mixture and the β-pepsin–digest mixture, using 10 and 25 transfer distributions, but were not investigated further.

It was found that the distribution coefficient increases with the concentration of complexing acid, but with TCA a sharp increase occurred between 0.2 and 0.3%. The complexing effect of TCA is sharply reversed by hydrochloric acid, while a gradual decrease in K occurs with increasing sodium chloride. It is interesting to note that sodium trichloroacetate gives a much higher K than TCA at 0.2% but a lower K at 0.5%. The effect of a small amount of this salt in acetic acid is rather surprising.

The water solubility of corticotropin was decreased tenfold by 3.6% NaCl; hence acetic acid was added to raise it to a practical level. As illustrated in Table II, the distribution coefficient increases with an increase in the concentration of either salt or acetic acid. The relation of the K's

TABLE I

DISTRIBUTION IN TRICHLOROACETIC ACID (TCA) SYSTEMS

1-BuOH[a] *vs.*	*p*H (aq. phase)	K[b] and sample
0.2% TCA[c]	2.0	<0.1, T(6–10)
.3% TCA[d,e]	1.7	*ca.* 8,T(6–10)
.5% TCA	1.7	*ca.* 13,T(6–10); 7, β
.5% TCA in 0.1 *N* HCl	1	0.6,T(6–10)
.5% TCA	1.7	2,P2 (*cf.* ref. 2)
.5% TCA in 0.01 *N* HCl	2	0.1,P2
1.0% TCA	1.4	*ca.* 5,δ_1
1.0% TCA in 0.06% NaCl	1.4	2,δ_1
1.0% TCA in 0.25% NaCl	1.4	0.7, δ_1
0.05% Na-TCA[f]	7	0.5,β
.2% Na-TCA	7	0.9,β
.5% Na-TCA[g]	7	1.2,β
.5% Na-TCA	9	0.3,β
6% HOAc	2.2	<0.02,T(6–10)
0.02% Na-TCA in 6% HOAc	2.2	0.6,β
.1% Na-TCA in 6% HOAc	2.2	*ca.* 13,β; 0.4, δ_1
Toluene·0.5% TCA (3:1:3)[c]	1.7	*ca.* 3,T(6–10)
1.0% F_3C–COOH[h]		*ca.* 2,T(6–10)

[a] 1:1 ratio unless specified; concentrations are g./100 ml. of solution. [b] Distribution coefficient K = organic/aqueous based on Nessler N or Folin protein analysis. [c] K varied considerably with slight temperature variation. [d] Mono- and dichloroacetic acid showed very dissimilar properties: K = <0.1 even at 12.5%. [e] 0.25% is like 0.3%. [f] Encountered difficulty with emulsions. [g] At 2%, K = *ca.* 10 for δ_1. [h] *Versus* 2-butanol; K increases with TFA concentration.

TABLE II

DISTRIBUTION IN SALT–ACETIC SYSTEMS

1-BuOH *vs.*	*p*H	*K* and sample
24% HOAc[a]	1.7	<0.02 β
1.6% NaCl in 6% HOAc	2.2	0.1 β; 0.05 γ_1
3.5% NaCl[b] in 6% HOAc	2.2	0.95 β; 0.3 γ_1
6.1% NaCl in 6% HOAc	2.2	1.0 γ_1; 0.25 γ_2
3.5% NaCl in 12% HOAc		0.8 γ_1; 0.24 γ_2
0.2% NaCl in 24% HOAc	1.7	<0.02 β
0.9% NaCl in 24% HOAc	1.7	0.5 0.9 1.7 in T(6–10)
3.5% NaCl in 24% HOAc	1.7	4 T(6–10); 0.2 δ_1
4.9% NaOAc[c] in 6% HOAc	4.4	0.3 T(6–10); 0.09 δ_1
4.9% NaOAc in 15% HOAc		1.0 β
4.9% NaOAc in 24% HOAc	3.7	>3 T(6–10)
1.3% NaOAc in 6% HOAc	3.7	<0.1 T(6–10)
8.2% NaOAc in 6% HOAc	4.7	<0.1 T(6–10); 0.2 δ_1
3% NaCl, 0.8% NaOAc in 6% HOAc	3.4	0.2 β; *ca.* 1 T(6–10)[d]
3% NaCl, 0.8% NaOAc in 24% HOAc	2.7	3 T(6–10)
1 *N* HCl in 18% HOAc		0.8 β

[a] Propionic was no better than acetic acid but isovaleric, lauric and salicylic acids give useful distribution coefficients. Concentrations are g./100 ml. solution. [b] At equal ionic strength, similar effects on *K* were found with NaI, LiCl, LiI, NH_4OAc and glycine; considerably lower *K*'s resulted with various polyvalent salts such as $CaCl_2$, NaH_2PO_4, Na_2SO_4, $MgSO_4$ or $Zn(OAc)_2$. [c] Other basic salts such as $NaHCO_3$, Na_2CO_3, Na_2HPO_4 or $Na_2B_4O_7$ gave much lower *K*'s. [d] Resolution of this mixture occurred in this system with 10 to 25 transfers.

for the α-, β- and γ-corticotropins remains about the same for the different systems. Preliminary investigations demonstrated that the T(6–10) mixture was fractionated by higher *p*H systems containing NaOAc but their possible utility was not further explored.

The *K*'s of the separated components of OC–corticotropin in 1-butanol *vs.* 3.5% NaCl in 6% HOAc are: α_1 12–14, α_2 4–5, α_3 1.5–2.0, β 0.95, γ_1 0.35, γ_3 0.27, γ_2 0.16 and δ_1 0.03.

Preparation of T(6–10).—Separation of OC–ACTH in the TCA system was so clean that it was usually accomplished on a large scale by a ten-tube separatory funnel operation, recovering the top phases of funnels 6–10 [T(6–10)] and the bottom phases of funnels 1–5 [B(1–5)]. Recovery was by the method described below for TCA systems. It was found later that the precipitate from a concentrated solution of OC–ACTH in 6% acetic acid, which was obtained by adding solid sodium chloride to 3.5%, was essentially equivalent to T(6–10). The B(1–5) fraction was entirely in the filtrate.

Countercurrent Distribution of ACTH.—This fractionation procedure[29a] was extensively used in this investigation. All countercurrent distributions were carried out in all-glass automatic machines having either 200 or 220 tubes sealed directly one to another without joints. Each tube was equipped with a sampling vent closed by a stopper. The air lock which was formed at this vent prevented contact of the liquid with the stopper. Two such machines accommodating either 13 ml. or 1 ml. of each phase were used. The tube design of the smaller of these machines is described in a succeeding paper.[2] Solvent from constant-level enclosed reservoirs was added automatically at the time of transfer by two dippers which led into the first tube. The first dipper added 13.0 ml. of top phase, and a second, which added 0.4 ml. of bottom phase, was required to maintain the phase volumes in the first few tubes. A ten-tube forerun of each phase preceded the charge tube to ensure solvent equilibration.

Determination of the optimum equilibration and settling times were carried out for each machine, with various types of samples and solvent systems. OC–ACTH was usually charged at 8–10 mg./ml. for distribution in the salt system; equilibration in the larger machine was achieved by a 5-minute shake period with 12 oscillations/minute, and the phases were clear after an 11- to 13-minute settle period. T(6–10) or β, charged at the same concentration, required a similar equilibration period, but cleared after a 5-minute settle time. In the smaller machine, employing a vigorous 30 oscillations/minute shaking, an equilibration time of $3^1/_2$ minutes was used. Further discussion of the effect of non-equilibrium is given in a succeeding paper.[2]

(29a) L. C. Craig and O. Post, *Anal. Chem.*, **21**, 500 (1949).

Preparation of Large Amounts of β.—Large quantities of β were prepared directly from OC–ACTH or T(6–10) by countercurrent distribution in the salt system above, charging in 40–60 tubes of the 200-tube machine in order to handle 3–4 g. The sample [3.0–4.5 mg./ml. for OC–ACTH; 7.5 mg./ml. for T(6–10)] was dissolved in 6% acetic acid equilibrated with 1-butanol and solid sodium chloride was added to 3.5%. Concentrations given here and throughout this work are g./100 ml. of solution. Since the final solution was saturated with ACTH, this method of solution minimized solubility difficulties. The solvent system was prepared by equilibrating equal volumes of 1-butanol and 3.5% sodium chloride in 6.0% acetic acid. The ACTH solution was charged in the apparatus and distribution was carried out, allowing the top phase to feed out into a fraction collector at the end of the machine. The separated α-, γ- and δ-fractions were removed after about 300 transfers and replaced with blank solvent. The machine was set to recycle the organic phase for an additional 200 transfers, at which point the remaining α- and γ-material had separated and was removed. The distribution of the β-peak was continued until a total of 984 transfers had been applied before it was considered of sufficient purity for further study. Recovery was by the procedure described for salt distribution systems. The yield of white β-acetate was about 35% of the charge.

A 2.5-g. sample of β (partially purified by 185 transfer distribution) was similarly distributed for 920 transfers. The peak was theoretical[29b] in shape (within experimental limitations) and the coefficients across the peak were constant within analytical error. The measured coefficients were $K_{10} = 0.89$, $K_{20} = 0.95$, $K_{40} = 0.93$, $K_{60} = 0.99$, $K_{80} = 0.96$, $K_{90} = 0.93$ (subscripts are tube numbers in machine after recycling up to $n = 920$). Redistribution of such material indicated a purity of about 95% with the contaminant being "esterified β."

Equally pure samples on a small scale can be prepared by 100- or 200-tube distributions of a single-tube charge and appropriate selection of the center of the peak for isolation. However, the "esterified β" was always present.

The specific optical rotation $[\alpha]^{23}D$ of β-corticotropin acetate hydrate was $-140°$ in 1 *N* HOAc containing 3.2% NaCl and $-150°$ in 0.1 *N* HOAc (*c* 0.5%).

Recovery from TCA Distributions.—Twenty equivalents of hydrochloric acid per mole of corticotropin (25.5 meq. per g. N) were added to the pooled, two-phase solutions and the mixture was equilibrated with 3 volumes of C.P. benzene. (The term "volume," as used throughout the description of recovery procedures, refers to the original aqueous phase volume.) The aqueous phase was removed and the butanol–benzene organic phase was extracted with 0.1 volume 0.01 *N* hydrochloric acid and 0.1 volume water, these extracts being added to the aqueous phase. The aqueous phase was then extracted twice, each with 3 volumes of C.P. benzene, washing the organic phases with two 0.1-volume portions of water at each step. The combined aqueous phases were treated with freshly washed Amberlite IRA-400 acetate to *p*H 3.5–4.0, the resin was removed by centrifugation and washed with water, and the supernate and washings were lyophilized to recover the white corticotropin acetate. Recoveries were greater than 90%.

Recovery from Salt Distribution Systems.—The recovery of these proteins from distribution systems containing salt was made possible by the fact that addition of TCA increased the distribution coefficient of ACTH far more than that of the sodium ion. As a result, a recovery procedure, essentially a four-stage countercurrent extraction, was designed to separate completely the sodium chloride and the ACTH, after which the corticotropins were recovered in the manner described for TCA systems. The method is described in detail below.

Two other recovery methods were investigated, one involving addition of hydrochloric acid and benzene to the original mixture and picric acid to the separated aqueous phase, and one involving adsorption of the corticotropin on oxycellulose and elution with 0.1 *N* hydrochloric acid. Neither was as satisfactory as the extraction procedure; the recovery through the picrate method was only 46%; the oxycellulose adsorbed large quantities of sodium ion which were eluted with the recovered product.

(29b) B. Williamson and L. C. Craig, *J. Biol. Chem.*, **168**, 687 (1947).

A. **Recovery of α- and β-Corticotropin.**—The aqueous phase from the pooled, two-phase solutions was made 0.5% in TCA by the addition of solid TCA in order to distribute the corticotropin into the organic phase. After removing the organic phase, the aqueous phase was extracted 3 times with 0.18 volume of 0.34% TCA in dry butanol and then was discarded. The organic phase was extracted 4 times, each with 1 volume of 0.17% aqueous TCA, these aqueous phases being back-extracted each time with 0.18 volume of 0.34% TCA in butanol.

To the pooled butanol extracts was added 0.25 volume of water and the mixture was treated with Amberlite IRA-400 acetate to *p*H 3.5–4.0. The aqueous phase was separated and 6 volumes of benzene or toluene and 0.00166 volume of concd. hydrochloric acid were added to the organic phase. This added step was required to recover any α-type material which, because of its greater organic-solubility, was not completely recovered without the addition of hydrocarbon solvents. The aqueous phase which separated from this extraction was removed and two 0.1-volume of 0.1 *N* hydrochloric acid washings of the benzene phase were added to it. These aqueous phases were treated with Amberlite acetate and combined with the previous *p*H 3.5–4.0 aqueous solution. Upon lyophilization the hormones were recovered as their acetate salts. The recovery averaged 80% and the product contained only 0.2% sodium chloride calculated from the flame photometer analysis for sodium.

B. **Recovery of γ-Corticotropins.**—The γ-corticotropins, having lower distribution coefficients than the β-material, were successfully recovered only if the TCA concentration was increased. For these fractions the original mixture was made 2% in TCA, and subsequent extractions of the aqueous phase were made with 1.36% TCA in 1-butanol. For extraction of the organic phase, water was satisfactory. In all other details, the procedure was identical to that described for α and β.

Corticotropin Assays.—In general, the Sayers assay[8] involved the use of 5–6 rats at each of 3 dosage levels. Since these assays were not designed to establish clinical potency, the U.S.P. standard was not usually run as a standard. "Clinical" ACTH had a potency of 1–2 U.S.P. units/mg. Oxycellulose ACTH and its separated components were approximately 100 times higher, with the exception of δ_1-corticotropin, which had about 10 U.S.P. units/mg.

Intermedin Assays.—The assay procedure is described in a succeeding paper.[3] Maximal melanophore expansion lasting 4–6 hours resulted from a dosage of 10 γ/100 g. of α_1, α_2, α_3, β, pool of γ_1 and γ_3, or pool of γ_2 and γ_3. The minimal effective dose of all these corticotropins was about 3 γ/100 g.

TABLE III

AMINO ACID ANALYSIS[a] OF CORTICOTROPINS

Amino acid	β molar ratio	Integer	T(6–10) molar ratio
Alanine	3.2	3	3.5
Arginine	2.9	3	2.8
Aspartic acid	2.1	2	2.0
Glutamic acid	4.7	5	5.0
Glycine	2.8	3	2.9
Histidine	0.9	1	0.7
Isoleucine	0	0	Trace
Leucine	2.0	2	2.2
Lysine	3.7	4	3.5
Methionine	1.0	1	0.7
Phenylalanine	3.1	3	2.9
Proline	4.3	4	3.9
Serine	1.8	2	1.5
Threonine	0	0	Trace
Tryptophan[b]	1.2	1	?
Tyrosine	2.0	2	1.8
Valine	3.2	3	3.0
Total NH_3 (by Dowex col.)	1.7[c]	1	2.8[c]
Amide NH_3	0.9[d]		

[a] Based on the average of two sets of columns per sample. [b] By procedure B of J. R. Spies and D. C. Chambers, *Anal. Chem.*, 20, 39 (1948). [c] Control runs demonstrated the liberation of nearly one mole of NH_3 due to general decomposition of the constituent amino acids during hydrolysis. [d] Method described.

Dowex Column Amino Acid Analysis.—The Dowex-50 method of Moore and Stein[22] was followed in detail. The sample was prepared by hydrolyzing in 6 *N* hydrochloric acid under nitrogen in a sealed tube at 110° for 18–24 hours. Results are given in Table III for both β and T(6–10).

Microbiological Analysis.[30]—Quantitative microbiological analysis of a T(6–10) hydrolysate, using *L. citrovorum* and *L. mesenteroides*,[31] established the L-configuration for all the amino acids except alanine and aspartic, for which the organisms were not specific, and methionine and tryptophan, for which assays were not carried out.

Elementary Analysis.[32]—The analytical sample was prepared by lyophilization of an acetic acid solution of β (*p*H 4.0) at 25° under 0.01 mm. pressure. Subsequent drying at 10^{-6} mm. and 100° resulted in the loss of 10.46% water, as shown in Table IV. A similar degree of hydration in other corticotropin preparations[26,33] is indicated by the low N analyses reported.

TABLE IV

ELEMENTARY ANALYSIS OF β-CORTICOTROPIN ACETATE

β-Corticotropin·32H_2O·2HOAc, $C_{214}H_{386}N_{56}O_{93}S$

	Found[a]	Found Corr. for 1.03% ash	Theory
C	48.37	48.87	48.83
H	7.29	7.37	7.39
N	14.70	14.85	14.90
H_2O	10.46	10.57	10.95
HOAc	(2.80[b])	(2.83)	2.28
Ash	1.03		
Cl	0.576		0.625[c]
C/N ratio	214.9/56.0		214/56

[a] Average of three determinations. [b] The acetic acid content was calculated by difference, using total carbon minus peptide carbon = acetic acid carbon. The peptide carbon was based on the peptide content calculated from the ratio of the found nitrogen to the nitrogen calculated from the empirical formula of the free base. [c] Assuming total ash is NaCl.

Analysis of β-hydrochloride, on a sample which had been dried to constant weight at 10^{-6} mm. and 100°, gave a confirmatory C/N ratio of 210.5/56.0 and revealed *stable* hydration (9.0%) in contrast to the acetate. The total water content of the lyophilized hydrochloride was 13.1% (based on C, N and Cl analyses, the peptide hydrochloride content was 86.9%).

Based on these elementary analyses and on the constituent amino acid analyses, the empirical formula of the free base of β-corticotropin is $C_{210}H_{314}N_{56}O_{57}S$ (molecular weight 4567.16).

Folin Protein Analysis.[34]—This rapid method, using an alkaline copper reagent, determines peptide nitrogen plus tyrosine phenol and tryptophan indole groups, but will not generally determine amino acids or small peptides. The color formation is sensitive to *p*H and a neutralized (within 0.03 meq.) sample is required. The TCA or acetic acid in a series of distribution samples can be conveniently neutralized by appropriately altering the amount of reagent 1 or increasing its sodium hydroxide content. Neutral salts (such as 3.5% sodium chloride) can be tolerated in the range of low protein concentration. The precipitation of the Folin reagent by organic solvents such as alcohols can be prevented by the addition of 2 ml. of cyclohexane/ml. of butanol without affecting color response.

Procedure for Butanol–Water Mixtures.—To the sample (1.0 ml. each phase) was added 2 ml. of cyclohexane.

(30) We are indebted to Mr. A. C. Dornbush of the Pearl River Laboratories for the microbiological assays.

(31) B. F. Steele, H. E. Sauberlich, M. S. Raynolds and C. A. Baumann, *J. Biol. Chem.*, **177**, 533 (1949).

(32) We are indebted to Dr. J. A. Kuck of the Stamford Laboratories, under whose direction the elementary analyses were performed.

(33) N. G. Brink, G. E. Boxer, V. C. Jelinek, F. A. Kuehl, Jr., J. W. Richter and K. Folkers, THIS JOURNAL, **75**, 1960 (1953).

(34) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol Chem.*, **193**, 265 (1951).

After thorough shaking, 5 ml. of reagent 1 (prepared by mixing 100 ml. of 2% Na_2CO_3, 1 ml. of 1% $CuSO_4 \cdot 5H_2O$ and 1 ml. of 2% sodium potassium tartrate) was added. After 10 minutes 0.5 ml. of 1.0 *N* Folin reagent was added. The solution must be rapidly mixed since the above authors report that the reagent has a half-life of 8 seconds at this *p*H. The optical density of the lower phase was determined at 6500 Å. (Beckman model DU spectrophotometer) after at least 30 minutes color development. If dilution of concentrated solutions is required, the diluent must be 1% Na_2CO_3, in order to maintain the correct *p*H (*ca.* 10.7). The extinction is increased by slightly lower *p*H and decreased (due to decomposition) at somewhat higher *p*H.

The method is sensitive to 40 μg. of OC-ACTH and an optical density of 1.0 is obtained with a 300-μg. sample.

Nessler Nitrogen Analysis.[35]—A micro Kjeldahl digestion was carried out in 150 × 18 mm. Pyrex tubes which had been photometrically selected as recommended by Moore and Stein.[36] The samples were dried in a nearly horizontal position in a vacuum oven at 65–70° overnight. They were then digested in 0.04 ml. of 9 *M* sulfuric acid[37] on an electrically heated sand-bath with a surface temperature of *ca.* 200°, and oxidized with 3 drops of 30% hydrogen peroxide (Merck superoxol). The peroxide treatment was repeated (usually once) until there was no color or charred material present. Without transferring, the samples were diluted with 5 ml. of water and then 0.6 ml. of Nessler reagent[37] (the gum ghatti was omitted), and the extinction at 4600 Å. was determined (Beckman model DU spectrophotometer), using hippuric acid as standard. The range of the method was 4–150 μg. of nitrogen. Larger amounts of nitrogen cause the formation of a precipitate.

Determination of N-Terminal Amino Acids by Dinitrophenyl (DNP) Substitution.—Early preparations of DNP derivatives were carried out as described by Sanger,[24] using 2.5% sodium bicarbonate in 50% ethanol and a 2-hour reaction time. However, 50% methanol was a better solvent for the basic pepsin digest materials and potassium bicarbonate was more soluble. Complete substitution was accomplished by using 5% potassium bicarbonate with a 4-hour reaction time and a 50-fold excess of dinitrofluorobenzene (DNFB).

The sample (2–10 mg.) was dissolved in 2 ml. of water and 1 ml. of 30% $KHCO_3$ was added. After adding a 50-fold excess of DNFB in 3 ml. of absolute methanol, the mixture was stirred for 4 hours at 25°. Without removing the precipitate the mixture was extracted with ether to remove DNFB. Acidification to *p*H 2 precipitated the DNP-substituted ACTH, which was washed with 95% ethanol and ether to remove dinitrophenol. The DNP derivative was hydrolyzed in 1:1 concentrated hydrochloric acid:glacial acetic acid at 105° for 24 hours. After evaporation, the aqueous solution was extracted with ether to remove the α-DNP amino acid, the amount of which was estimated by spectrophotometry of the yellow color (3475 Å.). The yield of DNP-amino acid from DNP-β-corticotropin was 0.6 mole with the conditions above. Quantitative paper chromatographic analysis of the non-extractable material showed complete loss of serine (2 moles) and considerable destruction (*ca.* 50%) of all the other amino acids. The possible presence of two N-terminal serines due to a tyrosyl serine ester link was excluded by the Edman degradation[3] of P2, P3 and T10 and by the results below of periodate oxidation and reductive methylation. DNP work with model peptides demonstrated that no peptide bond cleavage occurred during the reaction and that only the DNP derivative of the N-terminal amino acid was formed. The peptides investigated were the carbobenzoxy derivatives of L-Tyr-Gly-Gly·OH,[38] -Gly-L-Tyr·OH,[39] -Gly-Gly·OH,[40] -Tyr-DL-Ser·OH, the peptide H·Gly-DL-Ser·OH,[41] and the corticotropin fragments[3] C4 and T10. Quantitative analysis of the acid hydrolysates of their DNP derivatives (containing some by-product dinitrophenol) showed extensive decomposition of all the amino acids with serine decomposition the most extensive (70–80%). Therefore it seemed reasonable to conclude that the loss of the second mole of serine was due to decomposition during the hydrolysis of the highly dinitrophenylated β-corticotropin. This decomposition of serine may be accompanied by or be due to dinitrophenylation of its hydroxyl group.

Reductive Methylation.[42]—This end-group method is unique in not employing alkaline conditions. It involves dimethylation of amino groups at *p*H 5, using formaldehyde and hydrogen in the presence of palladium. When the reaction was carried out in aqueous alcohol, adsorption on the catalyst occurred with β and T10[3] but not with the model neutral peptide, H·Ala-Gly-Tyr-Leu·OH. The adsorption of the basic peptides was prevented by pyridine acetate.

Five mg. of β-ACTH was dissolved in 0.5 ml. of 0.25 *M* pyridine:0.2 *M* acetic buffer (*p*H 5) and 0.5 ml. of methanol; 0.1 ml. of 40% formaldehyde solution and 5 mg. of 10% palladium-on-carbon (J. T. Baker) were added. Hydrogen gas, saturated with the solvents, was bubbled through the reaction mixture. After 2 hours the amount of ninhydrin nitrogen had decreased to 5% and no adsorption on the catalyst had occurred, as measured by the diazonium coupling test.[43] After hydrolysis in 6 *N* hydrochloric acid, under nitrogen, for 18 hours at 105° the serine content had decreased from 2.0 moles to 0.7 mole, as measured by quantitative paper chromatography.[2] This established the presence of 1 mole of N-terminal serine prior to exposure to alkali and ruled out the presence of an aminoacyl ester linkage.

Periodate Oxidation.[44]—The sample (2×10^{-6} mole in 1 ml. of water) was placed in the outer chamber of a Conway cell and 0.5 ml. each of water, *p*H 5 pyridine–acetic acid buffer (1.25 *M*:0.62 *M*) and 0.02 *M* HIO_4 was added. Prior to the latter addition, the absorbing reagent was placed in the inner chamber. This reagent consisted of 100 mg. of chromotropic acid in 0.5 ml. of water and 0.5 ml. of concentrated sulfuric acid.

One set of samples was adjusted after 1.5 hours reaction to *p*H 1.3 to decompose aldimines. This set and another set run at the reaction *p*H (4.8) were allowed to diffuse for 18 hours at 25° (Table V).

TABLE V

FORMALDEHYDE LIBERATED BY PERIODATE

Sample	Mole of CH_2O/mole of sample Diffusion, *p*H 1.3	Diffusion, *p*H 4.8
CH_2O standard	0.43	0.62
Serine standard	.31	.41
OC-ACTH	.33	.37
OC-ACTH + CH_2O (1:1)	.78	.90

Although the recoveries of the standards averaged only 45%, the identity of the serine, formaldehyde and ACTH results warrants the conclusion that one mole of N-terminal serine is present in corticotropin, prior to any exposure to alkali.

Labile Amide and Total Ammonia Determination.—Initially it appeared from the analyses that β-corticotropin contained 2 moles of amide NH_3. However, work on the T10, T14, T17 and T1 fragments[3] of ACTH showed that, in each, general decomposition of the amino acids produced about 0.25 mole of ammonia (corrected for any free ammonia in the peptide). This decomposition (1–4%) thus accounts for what appeared to be a mole of ammonia in corticotropin in addition to the mole of labile amide. This "decomposition ammonia" was found in essentially this same amount in the degradation products[2] P3 and P4.

The amide group in β-corticotropin was found to be completely decomposed by 2 hours treatment with *p*H 10 borate buffer at 37° and with 6 *N* hydrochloric acid at 110°.

A rapid distillation procedure, rather than diffusion, was used to permit correction of the determination of labile

(35) G. L. Miller, *J. Exp. Med.*, **79**, 174 (1944).

(36) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(37) For the salt distribution system additional acid (0.12 ml. for 1 ml. each phase) was required to digest the residue. In these cases a larger amount of Nessler reagent (1.2 ml.) was added to maintain the correct *p*H.

(38) E. Abderhalden, *Fermentforsch.*, **16**, 110 (1938).

(39) M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **118**, 413 (1937).

(40) M. Bergmann, L. Zervas and J. S. Fruton, *ibid.*, **111**, 237 (1935).

(41) E. Fischer and H. Roesner, *Ann.*, **375**, 199 (1910).

(42) R. E. Bowman and H. H. Stroud, *J. Chem. Soc.*, 1342, 1349 (1950); V. M. Ingram, *J. Biol. Chem.*, **202**, 193 (1953).

(43) R. F. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," 2nd edition, Charles C. Thomas, Springfield, Ill., 1951, p. 445.

(44) *Cf.* ref. 39, p. 270.

amide for any free ammonia. One sample of peptide was distilled without pretreatment (free ammonia), another after brief hydrolysis at *p*H 10 (labile amide) and a third after total hydrolysis in acid. The method used was that reported by Archibald,[45] with a slightly modified apparatus. Preliminary work showed that distillation for 6 minutes under 30 mm. pressure in a 50°-bath was sufficient for 100% recovery of ammonia. At the completion of distillation, 0.6 ml. of the above Nessler reagent was added and the absorption at 4600 Å. was determined. The method was entirely reproducible in the range of 10–60 μg. of nitrogen and gave 98% recovery of standards.

Three identical samples of peptides were taken: one was distilled directly; the second was totally hydrolyzed under nitrogen in 6 *N* hydrochloric acid at 105° for 22 hours and dried prior to distillation. A stoppered third sample was injected with 0.5 volume (2 ml.) of *p*H 10 borate buffer and held for 4 hours at 37°. Then 0.5 ml. of 10% sulfuric acid was injected into the sample in order to reabsorb the liberated ammonia. After transferral to the distillation chamber, the additional acid was neutralized with 10% sodium hydroxide and distillation was carried out as usual.

Iodinated Corticotropin.—Five mg. of T(6–10) in 5 ml. of water and 5 ml. of 5% sodium bicarbonate was shaken with 2 ml. of carbon tetrachloride containing 1.13 mg. of iodine (10% excess over 4 moles of I_2/mole of peptide). Reaction was almost instantaneous. The aqueous layer was separated, made strongly acid (*ca.* *p*H 1) with 4 *N* hydrochloric acid and extracted three times with carbon tetrachloride to remove excess iodine. Treatment with Amberlite IRA-400 acetate to *p*H 3.0, followed by lyophilization, recovered a product of about one-fourth the corticotropin activity of the starting material. Evidence for completion of reaction was the failure of the product to take up any additional iodine, and the conversion to a single, new ninhydrin-positive component (R_f 0.66 on paper chromatography in 5:3:2 1-BuOH:H_2O:HOAc). This product had a strong ultraviolet maximum at 2800 Å. in 0.1 *N* hydrochloric acid and, in contrast to the starting material, was too insoluble for ultraviolet determination of ionic tyrosine phenol at *p*H 7.

Preparation of the radioactive derivative for distribution studies was similar.

Carbobenzoxy Corticotropin.—The carbobenzoxy derivative was studied because it was expected to have greater organic solubility and to be regenerable. Since its distribution properties were greatly changed from the unsubstituted corticotropin, countercurrent distribution of the derivative could provide an alternate fractionation. After a preliminary study of the reaction of carbobenzoxy chloride with lysine- and tyrosine-containing peptides, conditions were chosen which would provide selective reaction of the amino groups. Twenty mg. of T(6–10) in 5 ml. of water at 25° was adjusted to *p*H 9.3 with 0.1 *N* sodium hydroxide. Carbobenzoxy chloride (5.77 mg.; 8.5 moles/mole of peptide) in ether solution was added, and the reaction mixture was stirred for 50 minutes, maintaining the *p*H at 9.3 with 0.1 *N* sodium hydroxide. The solution, containing a small amount of precipitate, was extracted three times with anhydrous ether and acidified to *p*H 5.3 with 0.1 *N* hydrochloric acid to produce a white, gelatinous precipitate. This precipitate was washed with water, dissolved in 4.5 ml. of glacial acetic acid, diluted with 20 ml. of water, and lyophilized to give a 93% yield of the carbobenzoxy derivative (m.p. 194–199°). The product was inactive in the corticotropin and intermedin assays. The following data illustrate its distribution properties: methyl acetoacetate *vs.* water, K = 3.4; 1-butanol *vs.* 4.9% sodium acetate, K = 27; amyl alcohol *vs.* acetic acid, K = 1; and 1-butanol *vs.* 0.5% TCA, K = 23.

The carbobenzoxy derivative was regenerated[46] by dissolving in 1 *N* hydrogen bromide (22 equivalents/mole of protein) in glacial acetic acid and warming on a steam-bath for 5 minutes. A sample regenerated by this procedure had corticotropin activity, although the actual yield of activity was not determined. No activity could be regenerated by catalytic (palladium) hydrogenation, apparently because of adsorption of the protein on the catalyst.

Brief investigation of several benzylidine derivatives (benzaldehyde, *p*-nitrobenzaldehyde and 5-chlorosalicylaldehyde) showed that these also represented regenerable forms of the hormone with greater organic solubility.

Preparation of Deamidated β-Corticotropin.—Nineteen mg. of β-corticotropin was dissolved in 2 ml. of *p*H 9.0 sodium bicarbonate (0.1 *M*) buffer and charged on an Amberlite IRC-50 (XE-64) ion exchange column equilibrated with this buffer. The column was developed with additional buffer. A peak at two column volumes (140 ml.) accounted for 34% of the charge and a second resolved peak (unchanged β-corticotropin) at three column volumes for 46%. Total nitrogen or tyrosine recovery was 86%. The materials were recovered, after acidification to *p*H 4.0 with glacial acetic acid, by the procedure for salt distribution systems.

A second column was run with a charge that was allowed to stand at 25° in the *p*H 9.0 buffer for 22 hours prior to development. In this case the two peaks respectively accounted for 48 and 26% of the charge, with an 82% total recovery (nitrogen or tyrosine). The increase in the yield of the faster component with greater alkali-exposure demonstrates that this material is formed by an alkaline decomposition of β-corticotropin. Amino acid analyses and labile amide determination showed that the material differed from β only in having lost its amide group. Extension of the time of alkali-exposure to 2–3 days resulted in complete conversion to the deamidated material, which had the same corticotropin and intermedin activity as the starting β.

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STAMFORD, CONNECTICUT
PEARL RIVER, NEW YORK

[CONTRIBUTION FROM EXPERIMENTAL THERAPEUTICS AND MEDICINAL CHEMICAL SECTIONS, RESEARCH DIVISION, AMERICAN CYANAMID CO.]

Studies with Corticotropin. II. Pepsin Degradation of β-Corticotropin

BY P. H. BELL, K. S. HOWARD, R. G. SHEPHERD, B. M. FINN AND J. H. MEISENHELDER

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Three smaller active molecules containing 28, 30 and 31 amino acid residues, prepared from the 39-amino acid residue β-corticotropin by pepsin digestion, have been isolated in a homogeneous state. The relation of these active products is discussed in terms of the C-terminal peptide sequence.

One object of the investigation of corticotropin[1] was the preparation of a much smaller active molecule in order to make possible a practical synthesis. Such a molecule might, in addition, have more specific physiological activity than "clinical" grade corticotropin.

Peptide fragments were formed from purified hog corticotropin[1] by both enzymatic and chemical methods. Substantial cleavage leading to rapid inactivation[2] resulted with trypsin and chymotrypsin, and only a very limited degradation[1] with retention of corticotropin activity occurred with car-

(1) Studies with Corticotropin, I. R. G. Shepherd, *et al.*, THIS JOURNAL, **78**, 5051 (1956).

(2) Studies with Corticotropin, III, R. G. Shepherd, *et al.*, *ibid.*, **78**, 5067 (1956).