

amide for any free ammonia. One sample of peptide was distilled without pretreatment (free ammonia), another after brief hydrolysis at pH 10 (labile amide) and a third after total hydrolysis in acid. The method used was that reported by Archibald,<sup>45</sup> 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 pH 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<sub>2</sub>/mole of peptide). Reaction was almost instantaneous. The aqueous layer was separated, made strongly acid (*ca.* pH 1) with 4 *N* hydrochloric acid and extracted three times with carbon tetrachloride to remove excess iodine. Treatment with Amberlite IRA-400 acetate to pH 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<sub>f</sub>* 0.66 on paper chromatography in 5:3:2 1-BuOH:H<sub>2</sub>O: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 pH 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 pH 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 pH 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 pH 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<sup>46</sup> 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 benzylidene derivatives (benzaldehyde, *p*-nitrobenzaldehyde and 5-chlorosalicylaldehyde) showed that these also represented regenerable forms of the hormone with greater organic solubility.

**Preparation of Deamidated  $\beta$ -Corticotropin.**—Nineteen mg. of  $\beta$ -corticotropin was dissolved in 2 ml. of pH 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  $\beta$ -corticotropin) at three column volumes for 46%. Total nitrogen or tyrosine recovery was 86%. The materials were recovered, after acidification to pH 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 pH 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  $\beta$ -corticotropin. Amino acid analyses and labile amide determination showed that the material differed from  $\beta$  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  $\beta$ .

(46) G. W. Anderson, J. Blodinger and A. D. Welcher, *THIS JOURNAL*, **74**, 5311 (1952).

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(45) R. M. Archibald, *J. Biol. Chem.*, **151**, 141 (1943).

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## Studies with Corticotropin. II. Pepsin Degradation of $\beta$ -Corticotropin

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Three smaller active molecules containing 28, 30 and 31 amino acid residues, prepared from the 39-amino acid residue  $\beta$ -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<sup>1</sup> 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.

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

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

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

boxypeptidase. Dilute alkali removed only a single amide group<sup>1</sup> at 25° without affecting activity but quickly destroyed activity at 100° with only slight degradation.<sup>1</sup> On the other hand, pepsin treatment produced marked chemical changes with retention of activity except after prolonged exposure. Hot dilute acid formed a mixture in which the active molecule(s) had approximately the same distribution properties as the active pepsin digest products. This similarity was assumed to mean a closely related degradation (shown later to be correct<sup>2</sup>). Therefore, as between the alternatives of pepsin or acid for obtaining substantially smaller active molecules, the simpler mixtures resulting from the more specific action of pepsin were subjected to careful investigation.

Li and his colleagues<sup>3</sup> had found that mild pepsin or acid treatment of crude sheep corticotropin did not destroy biological activity. Li and Pedersen<sup>4</sup> later reported that the "average ACTH peptide" length in pepsin hydrolysates of the crude hormone corresponded to seven to nine amino acid residues. The "average molecular weight" of an inhomogeneous "ACTH peptide" fraction was subsequently estimated to be 2000 by Li, *et al.*<sup>5</sup> However, this concept of the average molecular weight is misleading, since such hydrolysates of pure hog corticotropin were mixtures whose components ranged in size from two to thirty-one amino acid units as shown in the present work. Furthermore, as reported herein, the activity resides only in the several larger molecules which are produced.

Early pepsin digestions of hog corticotropin demonstrated the need for a homogeneous substrate. For example, using oxycellulose-ACTH<sup>1</sup> or T(6-10) ACTH<sup>1</sup> as substrates, mixtures of six to eight groups of active components were produced. Subsequently the  $\alpha$ - and  $\gamma$ -corticotropins<sup>1</sup> were each digested to mixtures of about six components, and  $\beta$ -corticotropin to three. Thus, the pepsin digestion of this natural corticotropin complex presented the possibility of up to twenty-five to thirty different products.  $\beta$ -Corticotropin was selected for more detailed study since it produced a relatively simple mixture of three active molecules.

Various methods were briefly investigated for the separation of the pepsin digest products. However, it was soon found that several of the extraction systems listed in Tables I and II of the preceding paper<sup>1</sup> were suitable for countercurrent distribution and the components of such pepsin digest mixtures were thereby separated in a homogeneous state.

During the exploratory phases of the fractionation of corticotropin and of the pepsin digests, the need for distribution on a small scale became apparent. For this purpose a "micro" automatic countercurrent distribution apparatus using 1.0 ml. of aqueous phase and 1.0-1.5 ml. of organic phase was devised. The design proved satisfactory in all respects, and the value of such a machine was enormous. With this apparatus, extended dis-

tributions of small amounts of material were possible and it was practical to carry out exploratory distributions which could be easily scaled up for preparative work without changing the protein concentration.

The original trichloroacetic acid (TCA) precipitability of  $\beta$ -corticotropin disappeared completely after a few minutes exposure to a small amount (1/360 part) of pepsin. These mild conditions were studied in detail. Subsequent work showed that the digestion process was more complex and probably less specific at higher enzyme concentrations. The digestions were carried out with crystalline pepsin at 37° for 30 minutes in 0.01 *N* hydrochloric acid (*pH* 2). After enzyme inactivation by heating, TCA was added and the precipitate removed. Most of the TCA was extracted with ether and the *pH* was adjusted to 3.5-4.0 with Amberlite IRA-400 acetate ion exchange resin. Control experiments demonstrated the stability of  $\beta$  to the entire process in the absence of pepsin. Autodigestion controls failed to demonstrate any detectable amounts of amino acids or peptides arising from the enzyme itself at the levels used and showed that the pepsin was removed as the TCA precipitate during the work-up procedure.

A digest of  $\beta$ -corticotropin was prepared in this manner using an enzyme:substrate ratio of 1:360. In this and other experiments, the lyophilized product accounted for about 85% of the weight of the starting material, the remaining 15% being small inactive cleavage products which were removed by the Amberlite IRA-400 ion exchange resin. These small acidic peptides were recovered and their structures determined as described below.

Figure 1 shows an analysis of such a lyophilized digest after 197 transfers in 1-butanol *vs.* 0.5% TCA. Two peaks (*K ca.* 0.3 and 1.5) represent the major products. Extended distribution of the second major peak (*K ca.* 0.3) resulted in its separation (Fig. 2) into two apparently homogeneous materials,<sup>6</sup> P3 (*K* 0.23) and P4 (*K* 0.32). Recovery of the products as acetates was accomplished by the procedure previously described for TCA distribution systems.<sup>1</sup> Each of these three products was as active as the starting  $\beta$  in the Sayers corticotropin assay<sup>7</sup> and P4 was as active clinically<sup>8</sup> against rheumatoid arthritis. These new corticotropins, like the parent compound, have another hormonal action. As mentioned in the preceding paper,<sup>1</sup> pure  $\beta$ -corticotropin has a low level of intermedin activity which we regard as being due to the same peptide structure. This concept is considerably strengthened by the fact that the three pure active products, P2, P3 and P4, all have this same low level of " $\beta$ -type" intermedin activity. This type of intermedin is readily distinguished<sup>2</sup> and separated from the  $\delta_1$ -intermedin also present in crude ACTH.

(6) The nomenclature used in the preliminary reports (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)) of this work has been simplified here. Thus, the designation  $\beta$ Ps(2) of the previous reports becomes P2;  $\beta$ Ps(3) becomes P3, etc.

(7) M. A. Sayers, G. Sayers and L. A. Woodbury, *Endocrinology*, **42**, 379 (1948).

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

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

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

(5) C. H. Li, A. Tiselius, K. O. Pedersen, L. Hagdahl and H. Carstensen, *J. Biol. Chem.*, **190**, 317 (1951).

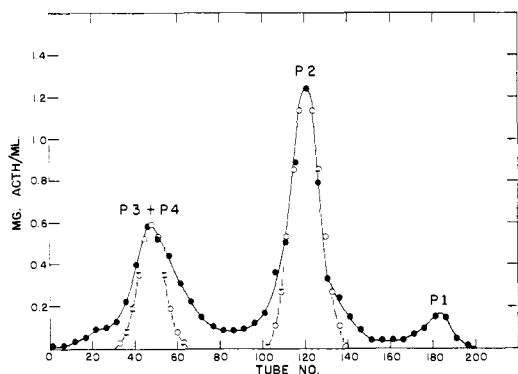


Fig. 1.—Distribution of pepsin-digested  $\beta$ -corticotropin in 1-butanol vs. 0.5% TCA, after 197 transfers (690 mg. charged in 3 tubes): o---o, theoretical curve.

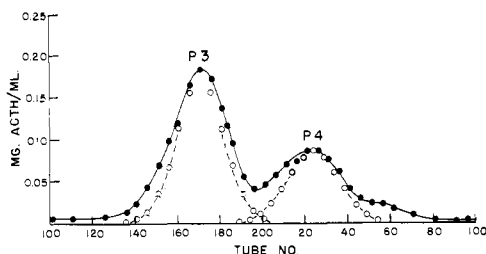


Fig. 2.—Continued distribution of P3 + P4 peak from Fig. 1 in 1-butanol vs. 0.5% TCA, after 921 transfers recycling top phase in the machine: o---o, theoretical curve.

The relation of these pepsin products to each other and to the parent  $\beta$  was determined by analysis of digests prepared with different amounts of enzyme and by redigestions of the isolated products. In preliminary experiments, variation of the enzyme:substrate ratio produced the components in the percentages shown in Table I. Analysis was by countercurrent distribution as above. Further work

Enzyme:substrate ratio	P1	Total nitrogen in peak, %	P2	P3 + P4
1:40	14	13	73	
1:120	15	33	52	
1:360	11	51	38	

showed that with the mildest digestion conditions (1:360 and less), the principal products were P2 (60%) and P3 (30%), with only about 6% of P4. With the more rigorous conditions (1:40 and greater) the digest contained less than 2% of P2 and correspondingly more P4. In contrast, P3 was present in approximately the same amount under both conditions. These results suggest that mild digestion gives P2 as the major product and P3 as the minor, and that P2 is the intermediate to P4. In order to substantiate the latter postulate, the presumed primary product P2 was redigested. Pure P2, after redigestion at a 1:120 enzyme:substrate ratio, gave the distribution results of Fig. 3. The new peak (30% conversion) appeared to be a single component since it was theoretical<sup>9</sup> in shape. The identity of this product and confirmation of its homogeneity was obtained from the fact that

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

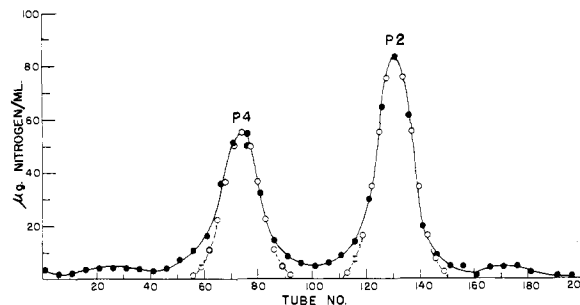


Fig. 3.—Distribution of redigested P2 in 1-butanol vs. 0.5% TCA, after 199 transfers (28.3 mg. charged in 1 tube): o---o, theoretical curve.

good integral molar ratios, correct for P4, resulted from its quantitative amino acid analysis.

The relation of the minor product(s) P1<sup>10</sup> to the others has not been proved. However, P1 is clearly not involved in the predominant process and comprised less than 2% of a pepsin digest of a purer sample of  $\beta$ -corticotropin. This decrease with greater purity suggests that it represents the amount of the "esterified  $\beta$ "<sup>11</sup> which was originally present as a contaminant.

The pepsin digestion data given above demonstrate that under mild conditions, there is an overall conversion of  $\beta$  partly to P2 and partly to P3, followed by a conversion of P2 to P4. This statement does not rule out any cleavages intermediate to these final products nor the possibility that other conversions, such as  $\beta$  to P4, P2 to P3, or P3 to P4, may occur to the extent of 10%.

It would be expected from the above results that more rigorous digestion of  $\beta$  would produce principally P4, along with some P3. However, pure P4 was prepared most readily by redigestion (1:120) of P2 since more rigorous digestion (1:40) of  $\beta$  gave variable results. This variation involved the formation of several other active products whose structures were not determined. However, their amino acid analyses indicated that they were not smaller molecules than P4 and that they represent alternative cleavages of the "non-essential" C-terminal sequence.

Other work on pepsin digestion of hog corticotropin<sup>11,12</sup> and sheep corticotropin<sup>5</sup> has not recognized the production of these several active molecules. None of our work revealed conditions leading to only one active product. On the contrary, the digestion conditions of Brink and co-workers<sup>11a</sup> produced in our hands a mixture of active components separable by distribution in the 1-butanol vs. 0.5% TCA system. In addition, the mixture of P3 and P4 was not resolved by the 2-butanol system which they used, and had the same distribution coefficient as their "Corticotropin-B." These facts leave in considerable doubt the homogeneity of "Corticotropin-B" and its identity

(10) This peak may contain a portion of the small acidic peptides if they are not completely adsorbed on the ion exchange resin. This adsorption was complete in control experiments but was not routinely checked.

(11) (a) N. G. Brink, F. A. Kuehl, Jr., J. W. Richter, A. W. Bazemore, M. A. P. Meisinger, D. E. Ayer and K. Folkers, *THIS JOURNAL*, **74**, 2120 (1952); (b) F. A. Kuehl, Jr., M. A. P. Meisinger, N. G. Brink and K. Folkers, *ibid.*, **75**, 1955 (1953).

(12) W. F. White, *ibid.*, **76**, 4194 (1954).



The molecule of  $\beta$ -corticotropin can thus be shortened by eleven of its thirty-nine amino acid residues without alteration of its corticotropin (and intermediin) activity. The formation of a smaller active molecule by pepsin degradation does not appear possible. However, subsequent work<sup>2</sup> with mild acid hydrolysis showed that bonds 24 and 25 are readily cleaved in a much shorter time than is required for inactivation. The resulting substances with twenty-four and twenty-five amino acid residues would appear to be, at present, the smallest active degradation products. Since these cleavages remove up to five of the six acidic side chains, it is surprising that activity is retained in spite of the resulting drastic change in the acidity of the molecule. The degradations of  $\beta$ -corticotropin reported herein seem to be the most extensive alteration of a natural hormone yet reported while retaining full biological activity.

### Experimental

**Micro Distribution Machine.**—Important factors in the design of tubes for this distribution apparatus were (1) small dimensions, (2) elimination of any "hold-up" after transfer and (3) a mixing chamber which could provide equilibration of phases in a short time interval. The tube (built to our design by Scientific Glass Apparatus Co.) sketched in Fig. 4 was satisfactory in all these respects when coupled with an automatic hydraulic drive mechanism which carried out a vigorous shake cycle. The exit section of each tube was sealed to its neighbor as indicated by the dotted circle, to provide a continuous all-glass circuit throughout. The tubes were designed as either left-transfer or right-transfer, to permit stacking several rows of tubes. A depth of four rows, 55 tubes per row, has been used satisfactorily here for 5 years without the occurrence of any breakage. The open end of each tube was closed by a cork and the air-lock thus formed prevented contact of the solvent and the cork. Top phase was added, at the time of transfer, by means of a dipper (volume 1.0–1.5 ml., depending on the depth of liquid). The very small amount (0.05 ml.) of bottom phase needed to maintain proper phase volume was added simultaneously by having a gently stirred mixture of the two phases (ratio about 6:1) in the closed constant-level reservoir.

The type of shaking and time required for proper equilibration were determined for this machine. The hydraulic shaking mechanism provided enough "snap" at the end of the forward motion of the tubes to empty completely the closed end of the equilibration chamber when shaking at 28 oscillations per minute, and 3 minutes at this speed was sufficient to accomplish equilibrium. We found that the effect of non-equilibrium was broadening of the peaks symmetrically with respect to the theoretical curve,<sup>9</sup> rather than skewing. For convenient emptying of the machine, a test-tube (13 × 40 mm.) was inverted over each tube, held in place by a rubber band, and the whole assembly was turned upside down (forward), allowing the contents of each distribution tube to flow into a separate test-tube.

This apparatus requires only 1.0 ml. each phase; thus a 220-transfer distribution can be adequately performed on as little as a 5–10 mg. sample, sampling 0.5–1.0 ml. each phase from every fourth or fifth tube for analysis. On the other hand, 100–200 mg. samples for extended (500–800 transfers) distributions could be handled by 3–6 tube multiple charging. In numerous "scaled-up" experiments carried out in the larger machine (13 ml. per phase) after preliminary distributions in the micro machine, perfect correlation of results was observed. The charging concentration of the acetates of the pepsin digests had no appreciable effect on the distribution in 1-butanol *vs.* 0.5% TCA in the range of 4–35 mg./ml.

**Pepsin Digestions.**—Digestions of  $\beta$ -corticotropin at an enzyme:substrate ratio of 1:360 were carried out as follows: a temperature-equilibrated solution of  $\beta$  (2 mg./ml.) was treated with  $1/180$  volume of freshly prepared pepsin (Worthington PM5, "twice crystallized from ethanol") solution<sup>14</sup>

(16) Prepared by finely powdering in an agate mortar and allowing to stand overnight at 25° in 0.01 N hydrochloric acid.

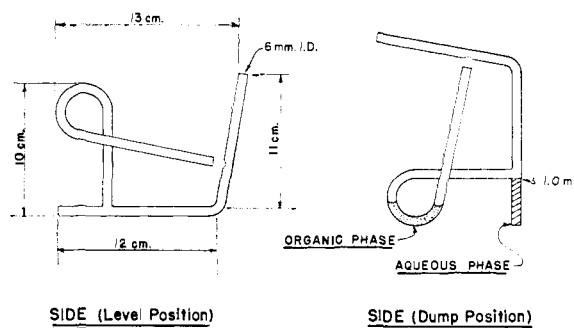


Fig. 4.—Tube design for "micro" countercurrent distribution machine.

(1 mg./ml.), both in 0.01 N hydrochloric acid. After 1 hour at 37°, the pepsin was inactivated by heating in a boiling water-bath for 30 minutes. The enzyme, along with any undigested  $\beta$ , was precipitated by the addition of one-fourth volume of 25% TCA. After standing in ice for 1 hour, the gummy precipitate was centrifuged. The supernate was extracted three times with anhydrous ether to remove most of the TCA. The rest as well as the chloride ion was removed by stirring for 1 hour with Amberlite IRA-400 acetate (80 mg./ml.) to adjust the pH to 3.5–4.0, and the digest products were recovered as their acetates by lyophilization.

Prior to distribution it was necessary to remove completely any chloride ion from the pepsin digests, since traces of chloride ion produced a sharp peak at the origin which was not found in distributions which were free of chloride. This effect was confirmed by the lowering of the distribution coefficient of  $\beta$  and its digests by the addition of hydrochloric acid or sodium chloride to TCA systems.<sup>1</sup> Acetate ion did not have this effect, and thus the acetate salts were suitable for distribution.

Distribution of 690 mg. of this pepsin digest (charged at 18 mg./ml. in three tubes) in 0.5% TCA *vs.* 1-butanol for 197 transfers (Fig. 1) resulted in the resolution of P2 ( $K$  1.53) in tubes 90–150. Recovery of the 108–130 section by the process for TCA distributions<sup>1</sup> gave 215 mg. of pure P2 acetate. The distribution of tubes 1–90 from this experiment was continued to 497 transfers, allowing top phase to feed out of the apparatus at tube 200. After analysis, tubes 80–150 were recycled in the apparatus to 921 transfers (Fig. 2), at which point P3 (tubes 135–195,  $K$  0.23) and P4 (tubes 1–50,  $K$  0.32) had separated. Pure P3 (63 mg.) was isolated from tubes 155–188, and 27 mg. of P4 from tubes 5–42.

Redigestion of 28.3 mg. of P2 at a 1:120 enzyme:substrate ratio gave 23.5 mg. of isolated acetate salt of the digest. This sample was distributed in 1-butanol *vs.* 0.5% TCA for 199 transfers. Undigested P2 (6.8 mg.) was isolated from tubes 120–140 and P4 (6.8 mg.) from tubes 62–83. The coefficient of P4 calculated from the position of its peak varied somewhat because of the transfer of a variable small amount (0.0–0.2 ml.) of bottom phase.

The behavior of  $\beta$ -corticotropin on pepsin digestion was much more satisfactory than that of T(6–10) in yielding fewer products which were more readily separated in pure form. The pepsin products of T(6–10) were broad non-homogeneous peaks which had distribution coefficients of 1.2, 1.7, 1.2 (II), 0.3 (III) and <0.1 in the 0.5% TCA system. The latter material was redistributed in 1-butanol *vs.* 2.0% TCA to give fractions with  $K$ -values of 5.8 (IV), 1.5 and 0.1. Four of these ( $K$  = 1.7, 1.2, 0.3 in 0.5% TCA;  $K$  = 5.8 in 2.0% TCA) were highly active. Digests of the  $\alpha$ -fraction<sup>1</sup> and the  $\gamma_1$ - and  $\gamma_2$ -fractions from OC-ACTH each showed the formation of four to six groups of products, only one of which had a distribution coefficient (0.3) common to all the fractions. These findings demonstrate that un-fractionated material, such as OC-ACTH or T(6–10) result in such complex digest mixtures that fractionation of starting material was essential.

Brink, *et al.*, reported<sup>11</sup> the isolation of a single highly active product in a homogeneous state by countercurrent distribution in 2-butanol *vs.* 0.5% TCA of pepsin-digested OC-ACTH. The inferiority of 2-butanol for resolution of pepsin digest mixtures is clearly shown by the

data of Table IV. The 1-butanol system resolved the components of pepsin digested T(6-10) with  $K$ -values as shown. These isolated components were then redistributed in 2-butanol to give the indicated  $K$ -values. The range of distribution coefficients is not nearly as great in 2- as in 1-butanol. Consequently, the peak maxima of III and IV

TABLE IV  
COMPARISON OF DISTRIBUTION COEFFICIENTS IN 1- AND 2-BUTANOLS

Solvent	Distribution coefficient of pepsin Digest fraction		
	II	III	IV
1-BuOH:0.5% TCA	0.9	0.3	0.06
2-BuOH:0.5% TCA	1.2	.7	.6

could only be 7 and 16 tubes apart after the reported 180 and 450 transfers, respectively. The peaks would overlap substantially in both these cases, and in the 20-transfer redistribution cited<sup>11</sup> as confirmation of homogeneity, they would be superimposed. In contrast, the 1-butanol system, after 180 transfers, would give almost complete separation of the peaks (29 tubes between the maxima). More significantly, the 1-butanol system was able to show that fraction III, even from the digestion of the highly purified  $\beta$ , was really a mixture of P3 and P4 ( $K = 0.23$  and  $0.32$ ), both of which have indistinguishable coefficients in 2-butanol.

**Pepsin Digestions at High Enzyme Concentrations.**—Such digestions were investigated enough to assure ourselves that no active molecules smaller than P3 and P4 were formed. The following experiments were analyzed by countercurrent distribution and the products indicated by quotation marks were characterized only by their distribution coefficients. In extended distributions, when the top phase was allowed to run out of the apparatus, the distribution coefficients were calculated using the formula<sup>17</sup>  $K = u/(n - u)$ , where  $u$  = the number of tubes in the apparatus and  $n$  = the number of transfers when the peak maximum left the apparatus.

In two digestions of  $\beta$ -corticotropin at a 1:38 ratio, only P3 and P4 were formed. When such a digest was distributed in 1-butanol *vs.* 0.5% TCA to 547 transfers, 30% of the charge was found to be P3 ( $K = 0.23$ ) and 50%, P4 ( $K = 0.32$ ). In another such digestion, on a different preparation of  $\beta$ , the products were "P3" ( $K = 0.21$ , 5%), "P4" ( $K = 0.32$ , 15%), PX<sub>2</sub> ( $K = 0.53$ , 16%), PX<sub>1</sub> ( $K = 0.69$ , 9%), PX<sub>3</sub> ( $K = 1.82$ , 15%) and "P1" ( $K = 7.7$ , 7%).

A similar production of at least one of these additional components was found on redigestion of P2 at high enzyme concentrations, whereas mild (1:120) redigestion of P2 gave only pure P4 (30% conversion). When P3 was digested under very rigorous conditions (1:1), the products, after 693 transfers, were "P3" ( $K = 0.19$ ), "P4" ( $K = 0.32$ ) and "PX<sub>2</sub>" ( $K = 0.54$ ) in roughly a 1:2:9 ratio.

The products PX<sub>1</sub> and PX<sub>2</sub> were approximately as active as  $\beta$ -corticotropin and therefore were examined to see if they represented smaller molecules than P4. PX<sub>1</sub> and PX<sub>2</sub> contained all the amino acids of P3, and PX<sub>1</sub> contained leucine, in addition.

**Dowex Column Amino Acid Analysis.**—The results of Dowex column amino acid analysis<sup>18</sup> of the pepsin digest products are given in Table V.

The following molecular formulas and molecular weights are calculated on the basis of the amino acid analyses and confirmatory structural data: P2, C<sub>185</sub>H<sub>254</sub>N<sub>48</sub>O<sub>45</sub>S, 3662.2; P<sub>3</sub>, C<sub>150</sub>H<sub>243</sub>N<sub>37</sub>O<sub>44</sub>S, 3549.0; P<sub>4</sub>, C<sub>150</sub>H<sub>229</sub>N<sub>44</sub>O<sub>38</sub>S, 3305.8.

**Ultracentrifuge Molecular Weight.**<sup>19</sup>—The data were interpreted using the Archibald<sup>20</sup> method. The observed molecular weight for P2 was 3550, which agrees well with the calculated 3662. For P3 the found value is 3330, compared with the calculated value of 3549, and for P4, 2670, compared with 3306. The experimental values were corrected for charge effects.

**Biological Activity.**—The corticotropin activity of the pepsin digest products P2, P3, P4, PX<sub>1</sub> and PX<sub>2</sub> was about

(17) L. C. Craig and D. Craig, "Technique of Organic Chemistry," Vol. III, Interscience Publishers, New York, N. Y., 1950, p. 289.

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

(19) We are indebted to Dr. R. A. Brown, of our Pearl River Laboratories, for this study.

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

TABLE V  
DOWEX COLUMN AMINO ACID ANALYSIS OF PEPSIN PRODUCTS

Amino acid	Molar ratios			
	Found <sup>a</sup> P3	Integer	Found <sup>b</sup> P4	Integer
Alanine	1.2	1	1.2	1
Arginine	3.3	3	2.7	3
Aspartic acid	2.0	2	1.2	1
Glutamic acid	2.8	3	2.1	2
Glycine	2.9	3	3.1	3
Histidine	1.1	1	1.0	1
Leucine	0	0	0	0
Lysine	4.0	4	3.8	4
Methionine	1.2	1	0.7	1
Phenylalanine	1.1	1	1.0	1
Proline	3.7	4	3.8	4
Serine	1.8	2	1.9	2
Tryptophan <sup>c</sup>	1.0	1	1.0	1
Tyrosine	2.2	2	2.0	2
Valine	3.1	3	2.9	3
NH <sub>3</sub> (by distn. <sup>d</sup> )	0.74	1	0	0

<sup>a</sup> One set of columns. <sup>b</sup> Average of two sets of columns. <sup>c</sup> By procedure B of J. R. Spies and D. C. Chambers, *Anal. Chem.*, **20**, 39 (1948). <sup>d</sup> Procedure described in ref. 1. Ammonia determination by Dowex column was high because of general decomposition (*ca.* 4%; *cf.* ref. 1) of the constituent amino acids.

100 units/mg. P1 was considerably less active (*ca.* 20 units/mg.).

These products were all active in the intermedin assay at about 5  $\mu$ g./100 g. of frog (minimal effective dose). Acid-boiled (0.1 *N* HCl, 4-16 hours) OC-ACTH, acid-boiled  $\beta$ -corticotropin and pepsin-digested OC-ACTH were all active at the same dosage both before and after adsorption and elution from oxycellulose. The unadsorbed material was inactive at a dose of 80  $\mu$ g./100 g. of frog, in each case. The pepsin digests of  $\gamma_1$ - and  $\gamma_2$ -corticotropin were active at less than 10  $\mu$ g./100 g. of frog. It is seen that in each instance the new product(s) has the same intermedin potency as the starting  $\beta$ -corticotropin. Deamidation<sup>1</sup> of  $\beta$  makes it *more* acidic, in contrast to pepsin or acid degradation, and here again the new product has the original intermedin potency (m.e.d. 3  $\mu$ g./100 g. of frog).

**Recovery of Small Acidic Peptides from Pepsin Digests.**—The Amberlite IRA-400 acetate, after use in pH adjustment of a pepsin digest, was slurried in water, poured into a glass column (0.6 cm. i.d.) and slowly eluted with 1 *N* hydrochloric acid (flow rate *ca.* 12 drops/min.). The course of the elution was followed by ninhydrin analysis of the effluent. The peptide material was eluted at between pH 5-2 and was recovered as a fluffy white solid by lyophilization; any material recovered from effluent cuts below pH 2 decomposed to black oils during lyophilization. The lyophilized peptides were separated by paper chromatography, as described below. This method was used for the peptides from digestion of  $\beta$  and from redigestion of P2.

**Paper Chromatography of Peptides.**—The successful countercurrent separation of the natural corticotropin complex in 1-butanol *vs.* 3.5% sodium chloride in 6% acetic acid<sup>1</sup> and of the pepsin digests in 1-butanol *vs.* 0.5% TCA suggested the use of paper chromatography for quicker analysis. However, paper chromatography using these systems failed due to the very low  $R_f$  values of all these basic peptides, presumably due to their strong adsorption by the cellulose.<sup>21</sup>

A similar strong adsorption was found to occur with the basic peptides arising from trypsin and chymotrypsin digestions<sup>2</sup> of  $\beta$ . With acidic peptides the magnitude of such effects, if observed at all, was very much less. It was possible, however, to obtain satisfactory separations of the basic peptides due to the displacement development of the peptides by each other. The  $R_f$  of the components of the

(21) Addition of pyridine to these solvent systems would possibly be beneficial, as found by S. G. Waley and J. Watson [*Biochem. J.*, **55**, 328 (1953); **57**, 529 (1954)] and by White and Landmann [*This Journal*, **77**, 1711 (1955)] for certain small basic peptides.

mixtures depended on the concentration of the other components and, after separation, the  $R_f$  of a single component varied with the amount charged. When larger amounts were separated by charging as a band on paper sheets, it was essential to keep the amount of charge per unit area constant. When charging was not uniform a series of wavy bands resulted. The bands on such chromatogram sheets were located as accurately as possible using the ultraviolet fluorescence, the ninhydrin color and intensity, and appropriate specific amino acid tests.

The peptide was applied as a  $44 \times 0.6$  cm. band on Whatman #1 filter paper ( $18.5 \times 22.25$  inches), charging 1 mg./cm. length. Development of these sheets was carried out for 16–54 hours in the appropriate solvent (usually the organic phase from 1-butanol:water:acetic acid, 5:4:1 by volume) without allowing an equilibration period in the tank before solvent addition. The peptide positions on the chromatogram were located by removal of small lengthwise strips from each end (and generally also from the center) of the sheet. One of these guide strips was sprayed with pyridine and 1% ninhydrin reagent as described below. Greatest color from peptides resulted from more heating ( $105^\circ$  air oven for 15–20 minutes) than is required for amino acids. The remaining guide strips were used for various specific amino acid tests.<sup>2</sup> The peptide bands on the main sheet were marked and cut out on the basis of the guide strips and any ultraviolet fluorescence of the untreated chromatogram. These bands were eluted with 0.1 *N* hydrochloric acid or 0.1 *N* acetic acid, depending on the peptide, in a manner similar to that reported<sup>22</sup> by Sanger and Tuppy. A general formula, sq. in. paper  $\times$  0.31 ml. collected eluate, corresponding to about four complete washings of the strip, was used to define complete elution. This was far more than enough for acidic peptides, but was required for the basic ones. The separated peptides were recovered by lyophilization.

The  $R_f$  values, as well as the amino acid mole ratios obtained after quantitative paper chromatographic analysis, of the acidic peptides of Table III are given in Table VI.

TABLE VI  
 $R_f$  AND AMINO ACID ANALYSIS OF SMALL PEPTIDE FRAGMENTS

Peptide	$R_f$ , 5:4:1 1-BuOH: H <sub>2</sub> O: HOAc	Cm. from origin after 64 hr. 2-BuOH: 3.0% NH <sub>3</sub> <sup>a</sup>	Molar ratios					
			Ala	Asp	Glu	Leu	Phe	Pro
PA2	0.75	11.0			0.9	1.3	1.0	0.8
PA1	.75	0.45	2.1		1.7	1.3	1.9	1.0
PA3	.67	2	1.8		2.2	1.3	1.5	1.0
PA4	.67	7	2.0		0.8	1.1	1.0	
PA5	.83		1.6		1.8	2.1	2.5	0.9
(1)	.71				0.9	1.0		
(2)	.55			1.1	1.0	1.0		
(3)	.22			+ <sup>b</sup>	+			

<sup>a</sup> System of J. F. Roland, Jr., and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954). <sup>b</sup> Not quantitatively determined.

**Quantitative Amino Acid Analysis by Paper Chromatography.**—Quantitative amino acid analysis of all peptide fragments obtained here and in the succeeding paper<sup>2</sup> was carried out by paper chromatography using ninhydrin color formation on paper and measurement after elution. Solvent systems were evaluated for their ability to separate the fifteen amino acid mixture of  $\beta$ -corticotropin and the best combinations were 1-butanol:water:acetic acid (5:4:1; all solvent ratios are by volume) followed by either *t*-butyl alcohol:methyl ethyl ketone:water:diethylamine<sup>23</sup> (40:40:20:4) or methanol:water:pyridine<sup>23</sup> (80:20:4), depending on the amino acid combinations. In the two-dimensional method described, all the amino acids were resolved except alanine-proline. Extensive variation of the "coloration" procedure and the ninhydrin reagent solvent was carried out without greatly improving the reproducibility or color response of the method described. An important advantage of the procedure below is its failure to determine any am-

monia arising from the peptide sample or through air contamination of the paper. A peptide sample of  $0.5\text{--}3.0 \times 10^{-7}$  mole was sufficient for analysis. The unknown hydrolysate was always run directly alongside standard amino acids which were grouped so as to provide unequivocal identification by position and color. The more rapid one-dimensional chromatography gave less variability and higher color responses than two-dimensional work. Various 1-butanol:water:acetic acid systems gave the highest and most reproducible color responses. The coloration procedure for one-dimensional and two-dimensional chromatography was identical.

The sample was prepared by hydrolyzing in 6 *N* hydrochloric acid under nitrogen in a sealed tube for 20–24 hours at  $110^\circ$ , followed by evaporation in a nitrogen stream and re-evaporation after the addition of water. The aqueous solution ( $1 \times 10^{-7}$  mole/0.01 ml.) was then applied (*ca.* 6 mm. diameter spot) to the paper in successive small (*ca.* 3  $\mu$ l.) additions from a drawn-out 0.1-ml. pipet (calibrated to 1  $\mu$ l.) and air-dried. Successive additions were made from the same pipet for totals of 0.005, 0.015 and 0.03 ml. for ordinary quantitative work.

For mixtures requiring two-dimensional development, a modification of existing two-dimensional techniques was employed in order to permit side-by-side standards to be run in both dimensions. The sample was spotted in one corner of an  $18.5 \times 44.5$  inch rectangle of Whatman #4 filter paper (two  $18.5 \times 22.25$  inch sheets sewed together with cotton thread) and several qualitative mixtures of amino acid standards were spotted alongside the charge nearer the long edge. Without pre-equilibration in the tank, the 1-butanol:water:acetic acid solvent was added. After about 24 hours of descending development (just before the fastest amino acid reached the seam) the strip containing the standards was cut off and sprayed with pyridine and then with ninhydrin, as described below. Based on this guide strip, the main sheet was cut at about  $R_f$  0.4 to leave tyrosine, valine, methionine, phenylalanine and leucine on one section and the slower amino acids on another. Corresponding qualitative standards were spotted in one corner of each section, on a line with the original charge, and the sections were re-developed in the second dimension. The *t*-butyl alcohol:methyl ethyl ketone:water:diethylamine system was used for the slow amino acids, without pre-equilibration, allowing 16 hours development. Immediately after removal of the section from the tank, a jet of steam from a laboratory supply line was played back and forth over it for 5 minutes. This treatment vaporized the amine, which, if not carefully removed, causes a variable dark background which can obscure the amino acid spots. To complete this removal, the strip was dried for 30 minutes at  $65^\circ$ .

The section containing the fast amino acids was redeveloped in the methanol:water:pyridine system for 8 hours (equilibrated 6–8 hours for convenience only). It was air-dried, and further dried at  $65^\circ$  for 30 minutes. This system had greater resolving power than the former one for these amino acids and, in addition, eliminated any problems of high background color.

Both sections were uniformly sprayed on one side with 1% ninhydrin (Dougherty Chemicals) in 1-butanol saturated with 0.05 *M* phosphate buffer (*pH* 7), air-dried completely, and heated 30 minutes at  $65^\circ$ . After the spots were cut out, the color was eluted for 20 minutes, with occasional shaking, in a test-tube containing 3 ml. of 75% aqueous acetone, and the absorption at 5700 Å. (Beckman model DU spectrophotometer) was determined.

Proline, which was imperfectly resolved from alanine, required a special technique, a modification of the Chinard<sup>24</sup> method. The proline and alanine spots were eluted together as above to determine alanine, since the proline yellow color caused no significant contribution to the alanine response at 5700 Å. The sample, containing the paper segment, was then dried on a steam-bath and 0.2 ml. of 80% phosphoric acid and 2.0 ml. of ninhydrin reagent were added. The capped tube was heated in a boiling water-bath for 1 hour, diluted with 5 ml. of propanol:water (1:1), and its absorption at 5150 Å. was determined. The presence of alanine does not interfere with this proline analysis.

The procedure for one-dimensional chromatography was similar except that Whatman #1 paper was used. If an

(22) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(23) R. R. Redfield, *Biochim. Biophys. Acta*, **10**, 344 (1953).

(24) E. J. Harfenist, *THIS JOURNAL*, **75**, 5529 (1953); F. P. Chinard, *J. Biol. Chem.*, **199**, 91 (1952).

acidic system was used, the strip was sprayed evenly with pyridine and air-dried prior to the ninhydrin spray.

Certain effects observed in paper chromatography using 1-butanol:acetic acid systems were exploited in devising a procedure for resolving the alanine, aspartic acid, glutamic acid, glycine, proline, tyrosine, valine mixture by **double development in one dimension**. A procedure for unequivocal identification and simultaneous quantitative analysis of the fragments of peptide P4Tl<sup>2</sup> was desired. In one-dimensional chromatography of corticotropin hydrolysates and of the above amino acid mixture, the resolution of the spots in these systems was better if no pre-equilibration of the paper was carried out. Separation of the spots by 5:2:2 1-butanol:water:acetic acid was affected by the distance of the charge from the solvent source, apparently due to variation of the solvent gradient acting on the charge. However, this effect, at its optimum, was not quite sufficient to separate this seven amino acid mixture well enough for quantitative analysis. In 5:2:2 1-butanol:water:acetic acid, glycine and glutamic acid had the same  $R_f$  and in 5:2:3 1-butanol:water:acetic acid, glycine and aspartic acid had identical  $R_f$  values, the other amino acids being separated in each system. The required separation was not possible with intermediate solvent compositions but was achieved by using successive development in one-dimension with these two solvents, owing to the different relative position of glycine. The two solvents, in the order above, were used (for ca. 18 hours each) with air-drying of the paper to remove the solvents. The sample and standards were spotted as hydrochlorides since better resolution was obtained with this salt than with hydrolysates which had been neutralized with  $NH_3$ . The color responses are given in Table VII.

TABLE VII  
COLOR RESPONSES OF STANDARD AMINO ACID MIXTURES

Amino acid	Two-dimensions $\log \frac{1}{0.1}$ $\bar{T}$ $\mu\text{mole}^a$	One-dimensions <sup>a</sup> $\log \frac{1}{0.1}$ $\bar{T}$ $\mu\text{mole}^b$	Double-development in one-dimension $\log \frac{1}{0.1}$ $\bar{T}$ $\mu\text{mole}^b$
Alanine	0.21	0.44	0.46
Arginine	.13	.28	
Aspartic acid	.10	.14	.18
Glutamic acid	.17	.43	.43
Glycine	.12	.19	.19
Histidine	.10	.23	
Leucine	.27	.47	
Lysine	.19	.37	
Methionine	.17	.43	
Phenylalanine	.09	.15	
Proline (Chinard)	.24	.14	.15
Serine	.16	.38	
Tryptophan		.20	
Tyrosine	.10	.20	.20
Valine	.25	.47	.46

<sup>a</sup> Solvent system: 1-butanol:water:acetic acid (5:4:1).

<sup>b</sup> Corrected for blank.

Each amino acid required a separate standard curve, owing to the different color response. These **standard curves** were obtained by running chromatograms at four concentration levels of each amino acid, without previously subjecting the amino acid to hydrolysis. The quantity of amino acid present in hydrolysates was computed from these linear responses. Blanks, which were very low (optical density 0.02–0.03 for two-dimensional runs involving the diethylamine system; less than 0.01 for all others), were de-

termined by eluting blank paper segments of size and position corresponding to the amino acid spots cut from the ninhydrin-sprayed chromatogram. The responses of 0.1  $\mu\text{mole}$  of each amino acid are given in Table VII.

Even though the color yields are low (9–25%) due to known side reactions,<sup>25</sup> these color responses are so reproducible that no simultaneous quantitative standards were required. The reproducibility is illustrated by comparing the color responses of the one-dimensional butanol:acetic acid procedures in Table VII. For mixtures of standard amino acids, using either one- or two-dimensional development, and single determinations at each of four concentration levels, the standard deviation of the experimental points from the straight line "standard curve" was ca.  $\pm 12\%$ . For Edman end-group analysis, standards were prepared by one-dimensional chromatography of single amino acids and the standard deviation of the experimental points from the straight line standard curve was ca.  $\pm 3\%$ , using duplicate determinations at four concentration levels. The volume measurement described above was used in both instances.

Certain of the amino acids presented stability problems when subjected to acid hydrolysis, even under nitrogen. The destruction of tryptophan was complete on hydrolysis in air, although 10–25% could be detected when hydrolysis was carried out under nitrogen. Thus the presence of tryptophan could be qualitatively detected in hydrolysates. Folin analysis<sup>1</sup> was used to determine the tryptophan content of tyrosine-free peptides. The recovery of tyrosine in three peptide fragments<sup>2</sup> (P4Tl, P3Tl and I) which contained aspartic acid was significantly reduced with respect to tyrosine in aspartic-free peptides<sup>2</sup> (II, C4 and Tl0), and hydrolysis under nitrogen improved the yield but did not result in theoretical recovery. Methionine in peptides was especially sensitive to destruction during hydrolysis in air. In P4 and two peptide fragments<sup>2</sup> (C7 and Tl0) from  $\beta$  the recovery of methionine from "air-hydrolyzed" samples was reduced to 1–9% of standard unhydrolyzed methionine, but it averaged 90% under nitrogen. This effect was not noted with methionine itself, nor in synthetic mixtures of amino acids and methionine.

The method was satisfactory for determination of molar ratios in various peptide fragments from ACTH. The average standard error of molar ratios<sup>2</sup> in fourteen peptides (containing 2–22 amino acid residues) from  $\beta$  was  $\pm 4\%$ , using 2–4 replicate determinations. The analyses of the parent peptide hormones were not as reliable as those obtained by Dowex columns, but they could have been improved by greater replication and by the refinements indicated below. There was often some discrepancy between the amino acid ratios obtained from the two sections of the two-dimensional chromatograms, although the ratios within each group were consistent. This difficulty could be avoided by always running quantitative standards simultaneously. The method proved very valuable, gave integral mole ratios and required less time and much less material than the Dowex column method.<sup>18</sup> If required, greater accuracy could be attained by utilizing more standard determinations in the preparation of the standard curves, improving the volumetric measurements, running simultaneous quantitative standards and increasing the number of unknown peptide replicates.

The average  $R_f$  values of the amino acids investigated were essentially in agreement with those obtained by Redfield<sup>23</sup> for the systems used for the second-dimension systems, and the values for the 1-butanol:water:acetic acid (5:4:1) system were very similar to those reported<sup>26</sup> for 4:5:1 1-butanol:water:acetic acid.

#### STAMFORD, CONNECTICUT

(25) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948); D. A. MacFayden and N. Fowler, *ibid.*, **186**, 13 (1950).

(26) R. J. Block, "Paper Chromatography Lab. Manual," Academic Press, Inc., New York, N. Y., 1952, p. 67.