Purification on Amberlite IRC-50: Procedure B.—A column 66 cm. high and 2 cm. in diameter was filled with buffered Amberlite IRC-50 as described under procedure A. One gram of a corticotropin-B concentrate of potency 80 u./mg. and which had been prepared from commercial acid–acetone powder was dissolved in 20 ml. of aqueous sodium sulfite solution (200 mg. of sodium sulfite/l.) and passed into the column at a rate of 7 ml./hour. The column was washed with 200 ml. of the sodium sulfite solution. One liter of 10% aqueous pyridine containing 500 mg. of sodium sulfite was passed through the column in 1.5 hours, followed by 2.5 l. of 10% aqueous acetic acid at the same rate. The acetic acid eluate and the following acidic eluants contained hydrogen sulfide. The column was placed in a cold-room (0°) overnight. It was then eluted with 450 ml. of hydrochloric acid (*p*H 2.0, 0.01 *N*), which removed 60 mg. of material of potency about 150 u./mg. The following fractions were collected with hydrochloric acid of *p*H 1.58 and isolated as described in procedure A: fraction 1 (150 ml.), 41 mg., 170 u./mg.; fraction 2 (150 ml.), 40 mg., 375 u./mg.; fraction 3 (675 ml.), 50 mg., 275 u./mg.

Batchwise Purification on Amberlite IRC-50.—A mixture of 5 g. of Amberlite IRC-50 which had been buffered by 2% of sodium hydroxide, 500 mg. of a 75-u./mg. corticotropin-B concentrate and 50 ml. of a 0.2 *M* sodium sulfite solution was stirred for four hours. The resin was collected on a filter and washed with three 40-ml. portions of 10% aqueous acetic acid. The resin was slurried three times into 40-ml. portions of 0.1 *N* hydrochloric acid with occasional stirring on the filter and then sucked dry. The hydrochloric acid filtrates were adjusted to *p*H 2.5–3.0 with IRA-400 on the carbonate cycle and lyophilized. The colorless solid product weighed 210 mg. The recovery of activity at a level of 150 u./mg. was 84%. When a pepsin digest (80 u./mg.) was treated in the same manner except that 10% aqueous pyridine containing hydrogen sulfide was used in place of a sodium sulfite solution, a product of 180 u./mg. activity was recovered in an 80% yield.

Purification with Oxycellulose.—A column 0.9 × 90 cm. was packed with fresh, washed oxycellulose (10.4% carboxyl) suspended in 0.1 *N* acetic acid. The sample of corticotropin-B to be purified weighed 600 mg. and had an activity of 100 u./mg. All of the solutions used in this procedure contained hydrogen sulfide, and all were passed through the column at a rate of 6 ml./hour. The sample was applied to the column in solution in 10 ml. of 0.1 *N* acetic acid. The column was washed with an additional 50 ml. of the same solvent, and then with 250 ml. of hydrochloric acid at *p*H 1.58. The material eluted to this point, about 350 mg., showed assay values of less than 20 u./mg. The most active corticotropin-B was then eluted by hydrochloric acid at *p*H 1.40, and collected in 5.6-ml. fractions with the aid of an automatic fraction collector. The tenth and eleventh 5.6-ml. fractions yielded 48 mg. of product active at 330 u./mg.; fractions 12 and 13 gave 21 mg. of activity 320 u./mg.; and fractions 13 and 14 gave 7.5 mg. with an assay value of 210 u./mg.

Although activities of 300 u./mg. or more were obtained in this experiment, the procedure more frequently gave products of not more than 200 u./mg.

Repeated Oxycellulose Treatment of 300 u./mg. Corticotropin-B.—An 80 × 0.9-cm. column was filled with oxycellulose (10.4% carboxyl) which had been suspended in 0.1 *N* acetic acid containing hydrogen sulfide. A solution of 194 mg. of a corticotropin-B fraction of activity 300 u./mg. was introduced into the top of the column. All of the eluting solvents contained hydrogen sulfide. All of the fractions were collected at the rate of 6 ml./hour, and with the aid of a drop-counting automatic fraction collector adjusted to take 5.0-ml. fractions. The column was washed with 30 ml. of 0.1 *N* acetic acid, yielding 6 mg. of material of low activity. Elution with 125 ml. of hydrochloric acid at *p*H 1.58 gave 9 mg. of essentially inactive product. Finally, the active corticotropin-B was removed with hydrochloric acid at *p*H 1.40. As may be seen from Fig. 1, some 60% of the weight of the starting material was recovered in fractions 9 through 13 at activities of the order of 300 u./mg.

RAHWAY, NEW JERSEY

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, MERCK & CO., INC.]

Pituitary Hormones. VI. The Purification of Corticotropin-B by Countercurrent Distribution

BY FREDERICK A. KUEHL, JR., MELVIN A. P. MEISINGER, NORMAN G. BRINK AND KARL FOLKERS

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The isolation of corticotropin-B from its concentrates has been accomplished by the countercurrent distribution technique. In the solvent system employed, *s*-butyl alcohol and 0.5% aqueous trichloroacetic acid solution, the trichloroacetate salt of corticotropin-B was characterized by a distribution coefficient (C_{org}/C_{aq}) of 0.5 to 0.6. This component of high adrenocorticotropic activity behaved as a pure substance in countercurrent distributions of 200 to 450 transfers. Despite this, some loss of biological activity in the isolation of the most potent material has been indicated. The presence of two other components in all corticotropin-B concentrates subjected to countercurrent distribution has been observed. One of these appeared to have a low order of adrenocorticotropic activity.

Several groups of investigators have employed the countercurrent distribution technique for the fractionation of corticotropin and corticotropin-B concentrates. A phenol–ether/water system when applied to a pepsin digest of corticotropin with activity 18 u./mg. gave a fraction of potency 120 u./mg. based upon total nitrogen.[1] Other workers subjected 20 u./mg. of corticotropin concentrates which had initially been purified by cellulose chromatography to countercurrent distributions with butanol or *s*-butyl alcohol/aqueous organic acid systems and in some cases secured products of assay values about 100 u./mg. The best purifications were achieved with picric, sulfosalicylic and benzenesulfonic acids.[2] A recent report[3] has described the use of a countercurrent distribution system prepared with 2,4,6-collidine and water which separated pepsin-treated material from either sheep or porcine sources into at least two components. In neither case was the increase in activity as great as that expected from the separation of solids.

The solvent systems mentioned and a number of others which have been tried with apparently less success had offered one or more serious disadvantages: emulsification, extensive destruction of adrenocorticotropic activity, unfavorable distribution coefficient, difficulty of locating the adreno-

(1) J. B. Lesh, J. D. Fisher, I. M. Bunding, J. J. Kocsis, L. J. Walaszek, W. F. White and E. E. Hays, *Science*, **112**, 43 (1950).

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

(3) F. H. Carpenter, G. P. Hess and C. H. Li, *ibid.*, **197**, 7 (1952).

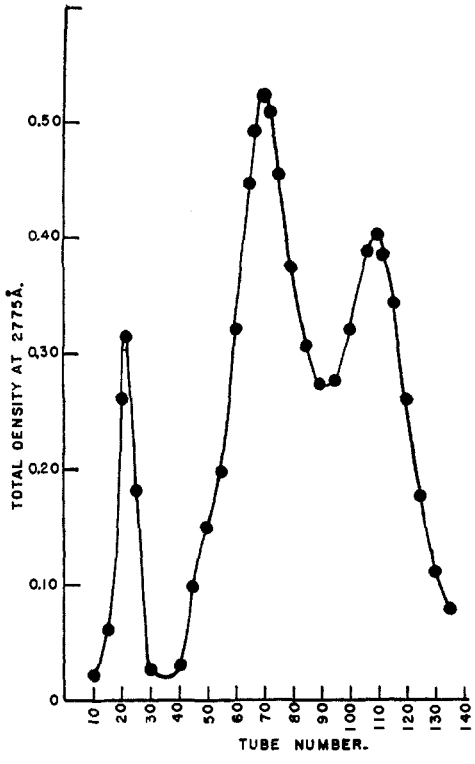

Fig. 1.—A 180-transfer countercurrent distribution of a 115-u./mg. concentrate of corticotropin-B.

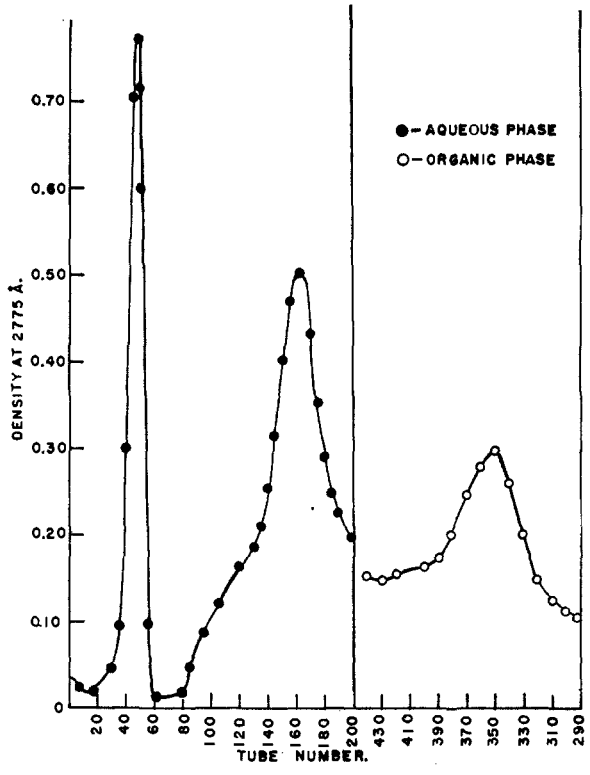

Fig. 2.—A 450-transfer distribution of a corticotropin-B concentrate of 300-u./mg. activity.. In the abscissa, tube numbers appear to the left of the vertical line, and transfer numbers to its right.

corticotropic material prior to isolation and difficulties in the isolation of the product. In the hope of finding a solvent system for countercurrent distribution of corticotropin-B in which these disadvantages would be minimal, a considerable number of distribution systems were examined. Of these, an *s*-butyl alcohol/aqueous trichloroacetic acid system appeared to be the most useful.[4]

The distribution system is prepared by equilibrating equal volumes of *s*-butyl alcohol and 0.5% aqueous trichloroacetic acid solution at 25°. Corticotropin-B has a favorable distribution coefficient ($K = C_{org}/C_{aq} = 0.5$–0.6 in this system) and both solvent phases are transparent to ultraviolet light in the 2775 Å. region where both corticotropin-B (and corticotropin) and its peptidic impurities absorb, permitting facile analysis of the fractions. In addition, this solvent system has shown no tendency to emulsify. Inactivation has occurred, especially with the most highly purified preparations; but it has been less troublesome than with any of the other distribution systems examined.

The countercurrent distributions were carried out in an all-glass apparatus[5] using 10 ml. of each phase per tube, and transferring the upper (organic) layers. When amounts of 250 mg. or less were to be distributed, all of the material was placed in the first tube; with more material, it was divided and put into two or more tubes. Since the preparations to be distributed were generally hydrochloride salts, an amount of sodium trichloroacetate equal to one-half the weight of the sample was added to the sample just before solution. At the end of the distribution, fractions were removed from the apparatus, the phases separated, and the contents of one or both phases determined by measuring the ultraviolet absorption of the solutions at 2775 Å. Products were recovered by passing both phases of appropriate fractions from the distribution over columns of the anion-exchange resin IRA-400 in the acetate cycle[2]; lyophilization of the eluates yielded white, amorphous acetate salts.

Corticotropin-B concentrates ranging in activity from 65 to 300 u./mg. have been examined by countercurrent distributions of 200 to 450 transfers. Figure 1 represents the results of a 180-transfer distribution of a sample of corticotropin-B which had given assay values of 115 u./mg.; a 450-transfer distribution of a 300 u./mg. corticotropin-B concentrate is depicted in Fig. 2. Both samples had been prepared from porcine corticotropin by pepsin digestion and purification on ion-exchange resins.[6,7] In both of these countercurrent distributions, and in all others which have been done, a major component characterized by a distribution coefficient of 0.5 to 0.6 was obtained. This fraction is corticotropin-B, and is the only fraction

(4) 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).

(5) The apparatus is described and illustrated in a chapter by L. C. Craig and D. Craig in "Technique of Organic Chemistry," Vol. III, Arnold Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1950, pp. 285–287. It was purchased from the H. O. Post Scientific Instrument Co., New York, N. Y.

(6) A. W. Bazemore, J. W. Richter, D. E. Ayer, J. Finnerty, N. G. Brink and K. Folkers, THIS JOURNAL, **75**, 1949 (1953).

(7) J. W. Richter, D. E. Ayer, A. W. Bazemore, N. G. Brink and K. Folkers, *ibid.*, **75**, 1952 (1953).

from the distributions which possesses high adrenocorticotropic activity. Both of the distributions represented in Figs. 1 and 2 revealed the presence of substantial amounts of two other components, characterized by distribution coefficients of K = *ca.* 0.12 and K = *ca.* 1.3. The nature of these two components will be discussed later.

For the examination of its purity, corticotropin-B was redistributed through twenty transfers in a steel Craig machine. A portion of the corticotropin-B isolated from tubes 64 to 73 of the 180 transfer distribution represented in Fig. 1 was used, and the customary *s*-butyl alcohol/0.5% aqueous trichloroacetic acid solvent system was employed. The results are shown in Fig. 3. Comparison of the observed and calculated[8] curves suggested an approximate purity of 95% for the sample of corticotropin-B examined. As a further criterion of purity, distribution coefficients based on the contents of adjacent pairs of tubes were calculated[8] and found to be equal within the experimental error for all adjacent pairs between fractions 4 and 9 as follows: $K_{4,5}$ = 0.53, $K_{5,6}$ = 0.55, $K_{6,7}$ = 0.53, $K_{7,8}$ = 0.52, and $K_{8,9}$ = 0.53.

The enhanced sensitivity of more highly potent samples toward a presumably oxidative inactivation and the minimization of the inactivation by the use of antioxidants have been reported.[4,7,9,10] When countercurrent distributions of some 200 transfers were carried out on corticotropin-B concentrates of potencies in the range from 65 u./mg. upwards, the purified corticotropin-B isolated in solid form as the acetate salt ordinarily gave assay values of 200 to 250 u./mg. However, in one experiment a sample of corticotropin-B purified by distribution to an activity of 200 u./mg. was increased in potency to 300 u./mg. by treatment with aqueous hydrogen sulfide and subsequent isolation by lyophilization. In another case, when the countercurrent distribution fraction containing the corticotropin-B component was processed through the Amberlite IRA-400 column, the column eluate was collected in a flask containing an aqueous solution of hydrogen sulfide weakly acidified with hydrochloric acid. Lyophilization of this solution afforded corticotropin-B with an activity of 300 u./mg.

When countercurrent distributions through 200 or 450 transfers of corticotropin-B concentrates, which had been purified by other methods to potencies of 200 to 300 u./mg., were done without the use of reducing agents, increases in activity paralleling the evident separation of solids (see Fig. 2) were not observed. This suggested that a significant inactivation was taking place in these final purification operations, either during the separation or in the isolation period. In order to study the distribution properties of inactivated material, a corticotropin-B concentrate was deliberately treated by mild oxidation to give a product of much lower activity; a portion of this was in turn reactivated by the use of hydrogen sulfide. Countercurrent distributions were done on material before and after the inactivation, and on the reactivated product. The observed distribution coefficients for all three samples were approximately equal. It may be concluded that "inactivated" corticotropin-B is indistinguishable from its active precursor on the basis of behavior in the distribution system studied. This observation may account for the difficulties frequently observed from the earliest stages in the purification of certain "poor batches" of concentrates. One might further expect that if this inactivation could be more completely prevented, a more highly active corticotropin-B could be obtained.

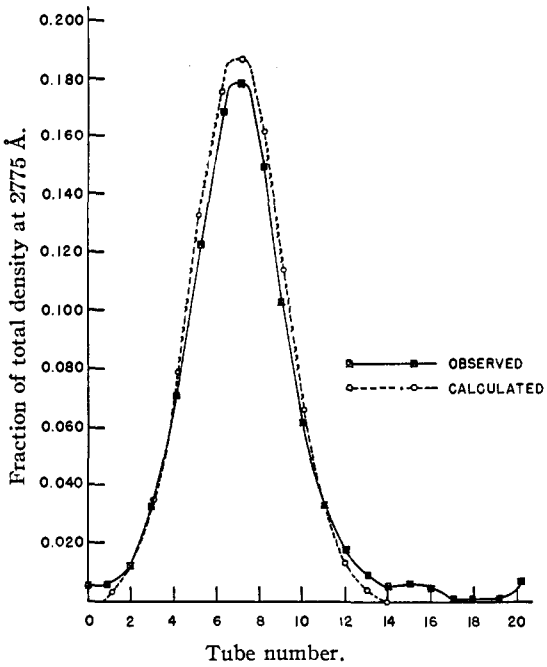

Fig. 3.—Analytical redistribution of corticotropin-B isolated from the distribution represented in Fig. 1.

The presence of two components other than corticotropin-B in concentrates purified by ion-exchange procedures has been noted. These have appeared in all preparations examined by extensive countercurrent distributions, although the amounts of each varied from one preparation to the next. In some experiments, one or both of these components have been isolated for examination as to intrinsic adrenocorticotropic activity and chemical nature. The more highly solvent-soluble component (K = *ca.* 1.3) in different preparations showed potencies ranging from 5 to 20 u./mg., but more extensive distribution appeared to decrease its activity. It is possible that the low order of activity exhibited by this component may simply have been due to contamination by small amounts of corticotropin-B. The water-soluble component (K = *ca.* 0.12) consistently gave assay values of about 20 u./mg., whether isolated from a 200- or from a 400-transfer countercurrent distribution. Although this component was probably active in its own right, it was less stable than corticotropin-B, and attempts to purify it further were difficult.

Differences in the behavior of corticotropin and corticotropin-B concentrates on the ion-exchange

(8) B. Williamson and L. C. Craig, *J. Biol. Chem.*, **168**, 687 (1947).
(9) H. B. F. Dixon, S. Moore, M. P. Stack-Dunne and F. G. Young, *Nature*, **168**, 1044 (1951).
(10) W. F. White, W. F. Fierce and J. B. Lesh, *Proc. Soc. Exptl. Biol. Med.*, **78**, 616 (1951).

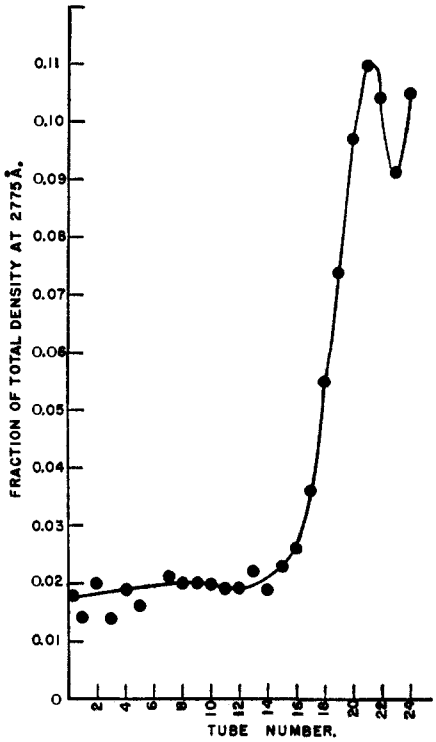

Fig. 4.—A countercurrent distribution of a 75-u./mg. corticotropin preparation which had not been digested with pepisn. The solvent system was *s*-butyl alcohol/0.5% aqueous trichloroacetic acid.

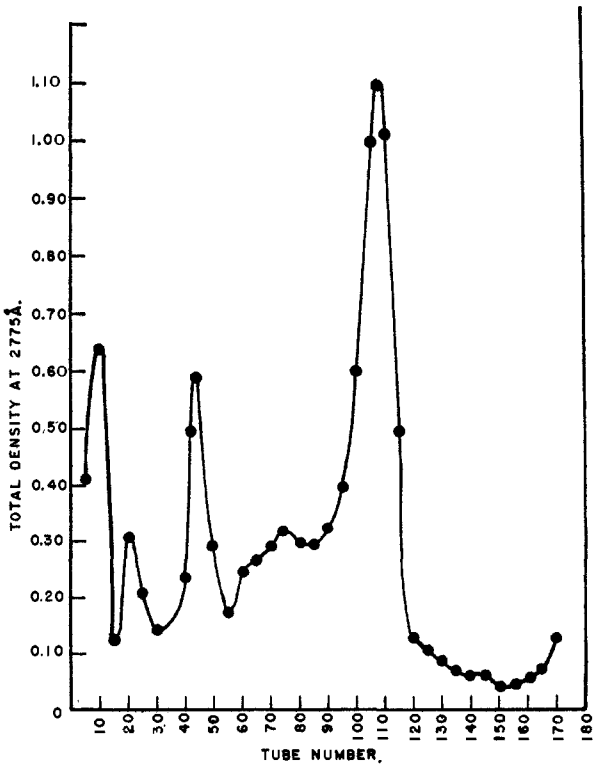

Fig. 5.—Distribution of a 100-u./mg. corticotropin sample in the modified solvent system *s*-butyl alcohol/0.1% aqueous trichloroacetic acid.

IRC-50, and the difficulties in purifying corticotropin beyond the 100 u./mg. activity level have been reported.[7] Corticotropin and corticotropin-B fractions were also clearly differentiated by their behavior in the *s*-butyl alcohol/0.5% trichloroacetic acid distribution system, the corticotropin fractions being extremely soluble in the organic phase. When a corticotropin concentrate of activity 75 u./mg. was subjected to a twenty-four transfer counter-current distribution in this system, most of the weight and activity was found in fractions 18 to 24. This distribution is represented in Fig. 4. Figure 5 shows the results of a 185-transfer countercurrent distribution of a sample of corticotropin of potency 100 u./mg. in a modified solvent system prepared with *s*-butyl alcohol and 0.1% trichloroacetic acid. Although separation of solids into three or four main components can be seen, no increase in activity resulted. The fraction with a maximum at tube 108 gave an assay value of 100 u./mg.; the other fractions had activities of 40–50 u./mg.

Experimental

180-Transfer Distribution (See Fig. 1).—The solvent system for the countercurrent distribution was prepared by shaking together 4-l. quantities of redistilled *s*-butyl alcohol and 0.5% aqueous trichloroacetic acid. The solvents were mixed and allowed to equilibrate in a room where the temperature was maintained at 25°, and where the distributions were subsequently carried out. The reader is referred to the recent review by Craig and Craig[5] for a photograph and description of the all-glass countercurrent distribution apparatus and for a detailed discussion of the technique. The machine was prepared for use by filling each of the lower tubes with aqueous phase (10 ml. per tube); ten to fifteen 10-ml. portions of organic phase were successively introduced into the machine in an effort to ensure thorough wetting of the inside of the apparatus. Finally, the first two tubes were emptied prior to introducing the sample.

The corticotropin-B sample to be distributed consisted of 300 mg. of material of potency approximately 115 u./mg., which had been fractionated on ion-exchange columns after pepsin digestion.[6,7] To it was added 153 mg. of sodium trichloroacetate, and the mixture was dissolved in amounts of the aqueous and organic phases which were adjusted to give 20 ml. of each phase after equilibration. Each of the first two tubes of the apparatus was then filled with 10 ml. of each phase of the solutions containing the corticotropin-B concentrate. The distribution was then carried out through 180 counted transfers; after each transfer, 10 ml. of organic phase was added to the first tube, and the phases were equilibrated by rocking the machine briskly with twenty up-and-down motions of the handle. The transfer, solvent addition, mixing and settling period permitted operation of the distribution at the rate of about fifteen transfers per hour.

At the completion of the distribution, the contents of every fifth tube were removed to numbered test-tubes and the phases separated with a pipet. The ultraviolet absorptions at 2775 Å. were then measured, using a sample of the organic or aqueous phases for a blank and adding a drop of methanol to clarify the solution in the cuvette. When necessary to locate the position of a maximum more precisely, additional fractions were removed from the apparatus and their contents analyzed spectrophotometrically. As may be seen from Fig. 1, major components were observed with maximum concentrations in tubes 21, 75 and 110, corresponding to distribution coefficients of 0.12, 0.6 and 1.2. The contents of several sets of tubes were removed from the machine and combined for isolation of the products.

Isolation of Corticotropin-B.—Products were isolated from the combined fractions 54–63, 64–73 and 74–78 of the above distribution by an ion-exchange process. The anion-exchange resin Amberlite IRA-400 was converted to the acetate form and used to fill columns about 1 cm. in diameter and 40 cm. high, which were then washed with *s*-butyl alcohol saturated with water. One such column sufficed to handle the contents of five tubes from the distribution apparatus. The solutions, consisting of both upper and

lower phases, were run through the columns at the rate of about 1 to 2 ml. per minute. The resin was then washed with water to displace residual solution. The eluates and washings were transferred to separatory funnels, shaken, and the organic layers discarded. The aqueous layers remaining were washed two or three times with *s*-butyl alcohol saturated with water, a process which removed traces of impurities introduced by the resins. The aqueous solutions were then lyophilized.

The combined fractions 54–63 gave 6 mg. of product, activity *ca.* 100 u./mg. Fractions 64–73 gave 18 mg. at a potency of *ca.* 230 u./mg. (average of several assays). Fractions 74–78 yielded 11 mg., which gave an assay value of 150 u./mg. in one determination.

Redistribution of Corticotropin-B.—A 16-mg. sample of the product of fraction 64–73 from the 180-transfer distribution described above and 8 mg. of sodium trichloroacetate were dissolved in 8 ml. each of the two phases of the *s*-butyl alcohol/0.5% trichloroacetic acid system. The material was put through a twenty-transfer countercurrent distribution, using a stainless steel Craig machine. At the end of the distribution, the contents of the aqueous phase from each tube was analyzed spectrophotometrically. The observed and calculated[8] distribution curves are shown in Fig. 3.

450-Transfer Distribution (See Fig. 2).—A 463-mg. sample of a corticotropin-B concentrate which had been purified to a potency of about 300 u./mg. by ion-exchange techniques[7] was used. To this was added 240 mg. of sodium trichloroacetate, and after solution in 20 ml. of each phase of the *s*-butyl alcohol/0.5% trichloroacetic acid system the mixture was placed in the first two tubes of the 200-tube all-glass apparatus. Four hundred fifty counted transfers were run. After 199 transfers, 10-ml. portions of the upper (moving) layer were withdrawn from the end of the train, assigned transfer numbers starting with 200, and stored for spectrophotometric analysis. Three main components, with maximum concentrations in tubes numbered 48 and 161–2 and transfer number 351 were found. The corresponding distribution coefficients were 0.12, 0.56 and 1.3.

Isolation of corticotropin-B acetic acid salt from tubes 158–167 gave 17 mg. of product of average assay value about 250 u./mg.

The Component of Distribution Coefficient 1.3.—Isolation of this component by the IRA-400 treatment as described for corticotropin-B yielded an amorphous white solid having the same appearance and general solubility properties as corticotropin-B. Preparations from a number of countercurrent distributions ranged in activity from 5 to 20 u./mg.

The Component of Distribution Coefficient 0.12.—This material was isolated from the distribution fractions where it occurred in the manner described for corticotropin-B. However, this acetate salt of the component was extremely hygroscopic. This effect was minimized by adding a slight excess of hydrochloric acid to the IRA-400 eluate before lyophilization. The hydrochloride so obtained was less hygroscopic and somewhat more stable. It consistently gave assay values of about 20 u./mg. when freshly prepared, although some samples lost activity on standing in a vacuum desiccator for a few days.

Countercurrent Distribution Behavior of Corticotropin.—After the addition of 13 mg. of sodium trichloroacetate, 23 mg. of corticotropin which had been purified by oxycellulose treatment and without pepsin digestion to a level of 75 u./mg. was put through a 24-transfer countercurrent distribution in a stainless steel Craig machine. The customary *s*-butyl alcohol/0.5% trichloroacetic acid system was used. The results of spectrophotometric analysis of the fractions after the distribution are represented in Fig. 4. It may be noted that most of the material appears at the extremely solvent-soluble end of the distributions, in fractions 18–24. Isolation by the usual method of the material in fractions 18–21 gave a product of activity 45 u./mg. The product from fractions 23–24 had a potency of 30 u./mg.

After some preliminary experiments had suggested that a solvent system containing less trichloroacetic acid might result in a more favorable distribution coefficient for corticotropin, a 400-mg. quantity of undigested corticotropin (100 u./mg.) was put through a 185-transfer distribution using the system *s*-butyl alcohol/0.1% trichloroacetic acid. Figure 5 shows the ultraviolet absorption of the various fractions. The isolated components showed no increase in activity: thus material from tubes 5–15 had a potency of 60 u./mg.; that from tubes 38–48 had a potency of 50 u./mg.; and the material isolated from tubes 101–115 gave assay values of about 100 u./mg. It should be noted that considerable difficulty with emulsions was encountered in working with corticotropin.

Inactivation and Reactivation of a Corticotropin-B Fraction.—One hundred milligrams of a corticotropin-B fraction which repeated assays had shown to have a potency of about 100 u./mg. was used. The sample was dissolved in 30 ml. of water, 1.5 mg. of ferric chloride was added, and the *p*H of the solution was adjusted to 7 with potassium hydroxide. A stream of air was bubbled through the solution overnight. The solution was treated with Amberlite IR-100 (H+) to remove metallic cations and was brought to *p*H 4 with Amberlite IRA-400 (carbonate cycle) and lyophilized. Repeated assays on the product demonstrated an activity of between 10 and 25 u./mg. One-half of the inactivated product was dissolved in 10 ml. of water and the solution was saturated with hydrogen sulfide and allowed to stand at room temperature for four days. After filtration to remove sulfur, the solution was lyophilized. Several assays of the product demonstrated that the reactivated material had a potency of approximately 100 u./mg.

Portions of the starting material, the inactivated product, and the reactivated product from this experiment were put through countercurrent distributions of 100, 50 and 50 transfers, respectively, in the usual *s*-butyl alcohol/0.5% aqueous trichloroacetic acid system. The observed distribution coefficients for the three samples were, respectively, 0.49, 0.52 and 0.56.

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