at 2775Å. Comparison of the observed and calculated curves[4] showed an approximate purity of 95%. As a further evidence of purity, distribution coefficients calculated on the basis of the contents of adjacent pairs of tubes[4] were: $K_{4,5} = 0.53$; $K_{5,6} = 0.55$; $K_{6,7} = 0.53$; $K_{7,8} = 0.52$; and $K_{8,9} = 0.53$. More recently, a countercurrent distribution of a highly purified corticotropin-B concentrate carried through 450 transfers in the *s*-butyl alcohol/trichloroacetic acid system revealed no indication of inhomogeneity in the corticotropin-B component.

It was early noted that the frequently observed loss of activity which has been most marked in highly purified preparations can be minimized by the presence of antioxidant during processing. Thus, corticotropin-B concentrates of 100 u./mg. activity were purified on oxycellulose columns to give material of the order of 200 to 300 u./mg. activity, provided that all solutions contained hydrogen sulfide. In its absence, the products had activities no greater than about 200 u./mg. Activities of *ca.* 300 u./mg. were observed for samples of corticotropin-B purified by distribution, when they were exposed to aqueous hydrogen sulfide solution prior to assay. Without this step, the material showed assay values in the range of 200–250 u./mg. In no case were antioxidants used in making up assay solutions of the solid products reported here. Others have recently reported[5,6] the use of reducing agents to prevent inactivation prior to assay.

Corticotropin at an activity level of about 100 u./mg. was clearly differentiated from corticotropin-B by its entirely different solubility behavior in the countercurrent distribution system, the non-digested material being excessively soluble in the organic phase.

The corticotropin-B described here has shown no evidence of inhomogeneity by the criteria of countercurrent distribution behavior which were applied. Further examination of the absolute purity of corticotropin-B is in progress, together with studies on its chemical, physical and biological properties, to be reported later.

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PURIFICATION OF ADRENOCORTICOTROPIC HORMONE BY CELLULOSE COLUMN CHROMATOGRAPHY[1]

Sir:

Since the introduction, by Astwood, *et al.*,[2] of oxycellulose for the batchwise purification of adrenocorticotropic hormone (ACTH), attempts to utilize this adsorbent in a column procedure have been unsuccessful,[3] probably because of the slow rate of attainment of equilibrium in such a system. Nevertheless, it is possible to employ such columns to obtain a reproducible purification by working under *non-equilibrium* conditions, where it is required that the amount of material placed on the column, the quantity of adsorbent, the column length and the rate of flow be carefully controlled.

In a typical experiment 10 mg. of purified sheep ACTH Preparation E[4] was pressed into a column (7 × 115 mm.) containing a mixture of 200 mg. oxycellulose[5] and 600 mg. cellulose powder,[6] washed according to the procedure of Astwood, *et al.*[2] Development was accomplished by means of a discontinuous *p*H gradient with 40 ml. 0.3 *N* $ClCH_2COOH$, followed by 40 ml. 0.7 *N* $ClCH_2COOH$, and finally by 20 ml. 0.1 *N* HCl. The flow rate was maintained at 4 ± 1 ml. per hour. The distribution of emerging material was obtained by the method of Lowry, *et al.*[7] Three peaks are obtained from such an analysis (Fig. 1). The average distribution of material in these three peaks, as

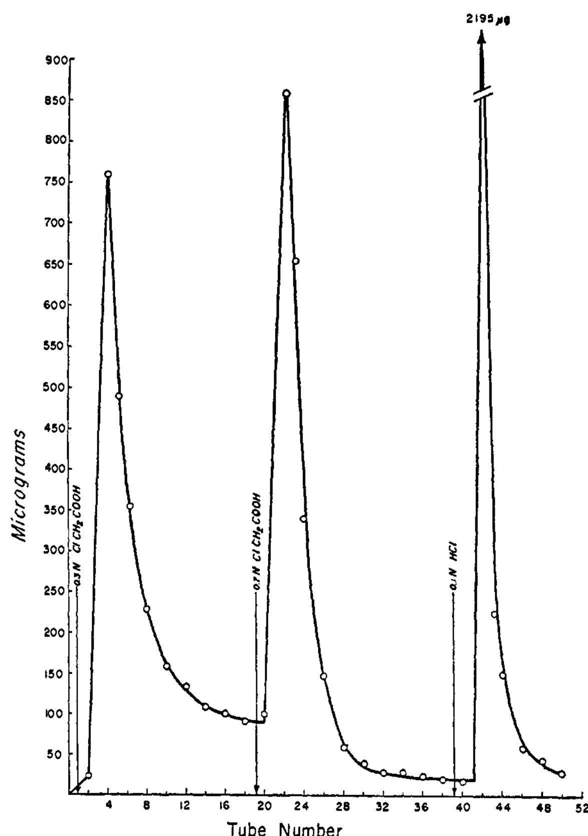

Fig. 1.—Chromatography of 10 mg. of an ACTH Preparation E (L2287E). The flow rate was 4 ml. per hour and the effluent was collected in 2 ml. fractions.

(1) This work is supported in part by grants from the U. S. Public Health Service, the Eli Lilly Laboratories, Merck and Company, Inc., the Armour Laboratories, and the Rockefeller Foundation.

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obtained from many experiments, is 41% in the first, 31% in the second, and 28% in the third peak. Assay of each of these fractions by the procedure of Sayers, *et al.*,[8] reveals the ACTH activity to be concentrated almost completely in the second peak. Thus a 2- to 4-fold purification is achieved (potency approximately 160 times standard). An oxycellulose-purified pig ACTH preparation,[9] when submitted to this type of analysis, revealed a distribution of 33, 54 and 13% in the first, second and third peaks, respectively.

The evidence for non-equilibrium in the system described is deduced mainly from the fact that the substances present in the first two peaks can be displaced almost completely to the last peak by means of flow rates which are much slower than those reported above.

Purification of Preparation E was followed by filter paper electrophoresis[10] and by two-dimensional paper chromatography of complete hydrolysates of the material in each of the peaks.[11] At *p*H 8, electrophoresis demonstrated that the first peak contained all of the anionic component(s), and the last peak the strongly cationic component(s). The middle peak showed a distribution of material at the origin or near it on the cathode side, areas where the activity has been found previously under these conditions.[4] Amino acid analyses reveal the first peak to contain all the amino acids found in the original preparations. The second peak contains only lysine, aspartic and glutamic acids (probably as amides), glycine, alanine, valine, leucine, phenylalanine, proline, and arginine (?). The third peak contains a large amount of arginine in addition to the amino acids found in the middle peak. Because of the high ultraviolet absorption of all fractions, tryptophane is presumed to be present in all three.

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(11) We are indebted to Dr. A. L. Levy for the amino acid analyses.

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THE EFFECTS OF DIVALENT CATIONS ON THE ENZYMATIC ACTIVITIES OF TRYPSIN AND OF α-CHYMOTRYPSIN[1]

Sir:

In view of current efforts to elucidate the mode of action of trypsin and α-chymotrypsin on synthetic substrates and inhibitors,[2] we wish to call attention to the fact that certain divalent cations which stabilize trypsin[3] also increase the enzymatic activity of trypsin and α-chymotrypsin.

The trypsin-catalyzed hydrolysis of 0.005 and 0.01 *M* benzoyl-L-arginine ethyl ester[4] at *p*H 7.8 and 25° is appreciably accelerated by some divalent cations, unaffected by others, whereas others again cause substantial inhibition. Twice recrystallized trypsin (Worthington, lot T-340), was dialyzed against 0.001 *M* HCl until salt-free, and lyophilized. The effects of divalent cations, added either as chlorides or sulfates, were tested in the presence of 0.005 *M* tris-(hydroxymethyl)-aminomethane·HCl buffer (THAM). Zero order reaction kinetics was observed in every case.

It is evident from the table that Ca^{++}, Mn^{++}, Cd^{++} and Co^{++} are most effective in increasing the activity of trypsin (by about 30%), Mg^{++} and Ba^{++} being somewhat less effective. The activation by Ca^{++} or Co^{++} is completely reversed by ethylenediamine tetraacetate (Versene). Trypsin is inhibited, in order of increasing effectiveness, by Zn^{++}, Hg^{++} and Cu^{++}. After partial inhibition by Cu^{++} (in borate buffer) partial reactivation by Ca^{++} can be brought about. The activity of Cu^{++}-inhibited trypsin (0.0005 *M* Cu^{++}) can be restored by Versene (0.001 *M*) and further activation is brought about by the subsequent addition of Ca^{++} (0.01 *M*).

TABLE I

EFFECT OF DIVALENT CATIONS ON THE HYDROLYSIS OF BAEE BY TRYPSIN

Cation added	Relative activity[a] 0.001 *M*	0.01 *M*
None	1.00	
Ca^{++}	1.28	1.28
Mn^{++}	1.34	1.35[b]
Cd^{++}	1.34	
Co^{++}	1.18	1.30[b]
Mg^{++}	1.09	1.26
Ba^{++}	1.08	1.28
Sr^{++} or Ni^{++}		1.10
Fe^{++} or Pb^{++}	About 1.0	
Zn^{++}	0.81	
Cu^{++} (borate buffer)	0.0	
Hg^{++}	0.0	
Versene, 0.01 *M*	1.00	
Co^{++} + Versene (0.012 *M*)	1.00	
Ca^{++} + Versene (0.012 *M*)	1.00	

[a] Activity expressed as ratios of zero order reaction constants in the presence of the added cation to that observed in 0.005 *M* tris-(hydroxymethyl)-aminomethane·HCl buffer, *p*H 7.8, alone. [b] Corrected for effect of partial precipitation of the metal oxide on alkali consumption during ester hydrolysis.

When these divalent cations were tested for their effect on the α-chymotrypsin catalyzed hydrolysis of 0.01 *M* acetyl-L-tyrosine ethyl ester[5] in 0.005 *M* THAM buffer, *p*H 7.8, only Ca^{++} produced significant activation. Maximum activation of about 50% was attained in the presence of 10^{-2} *M* Ca^{++} and one-half of that value was attained in 2×10^{-4} *M* Ca^{++}. In 0.001 *M* solutions, Zn^{++} yielded 50% inhibition, and Cu^{++} and Hg^{++} complete inhibition. α-Chymotrypsin completely inhibited by 10^{-3} *M* Cu^{++} could be partially reactivated by

(1) This work was supported by grants from the Public Health Service, the Rockefeller Foundation and the Lilly Research Laboratories, Eli Lilly and Company.

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