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The Theory and Use of Elution Analysis in the Partition Column Chromatography of Insulin between 2-Butanol and Dichloroacetic Acid, Hydrochloric Acid Solvent Systems[1]

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Preliminary studies on the partition column chromatography of insulin in solvent systems composed of 2-butanol equilibrated with 0.5% dichloroacetic acid in various concentrations of dilute hydrochloric acid, in which the aqueous phase was used as the stationary phase, are described. Anomalous results experienced in some solvent systems were attributed to a change in the composition of the solvent system resulting from the addition of the solute to the column. Such anomalies were avoided by the addition of the insulin to the column in an ionic form which was compatible with the solvent system. Results of the latter experiments indicated the presence of at least two components in the eluted fractions of the insulin samples studied. Equations are presented from which the performance of the column and the distribution constant of the solute can be determined from measurements made on the fractions eluted from the column.

In connection with investigations performed in this Laboratory on the purification of adrenocorticotropic hormone, it was noted that information obtained from countercurrent distribution (CCD) experiments was extremely useful in the design of experiments for the partition column chromatography of this material.[2] In theory, the partition column introduced by Martin and Synge[3] and the CCD technique of Craig[4] function on nearly the same principles. In practice, partition column chromatography experiments usually can be conducted with considerable savings in time and equipment over comparable experiments performed by CCD. Also, in CCD one frequently encounters solvent pairs which, on the basis of the distribution constant of the solute between the pairs, would be ideally suited for the technique but which have to be abandoned owing to the formation of emulsions that are slow to separate. The presence or absence of stable emulsions is of little importance in the selection of solvents for the partition column technique.

Presented in this paper are initial studies on attempts to compare the behavior of insulin in partition column chromatography with that reported by Harfenist and Craig[5] on the behavior of insulin in CCD. In order that a direct comparison could be made between the results of the partition columns and those of CCD, equations were derived which express the results of the columns in terms comparable with those used in CCD. These equations were developed by combining the mathematical treatment for the "single withdrawal" procedure of CCD[6,7] with the "theoretical plate" concept of chromatography introduced by Martin and Synge[3] and further developed by others.[8,9]

Although Porter[10] has subjected insulin to partition column chromatography, the solvent systems he used were not comparable with those used in CCD and apparently failed to differentiate between the A and B fractions of insulin observed by Harfenist and Craig.[5] In order to permit a more direct comparison of the results, solvent systems similar to those used in CCD experiments (2-butanol–1% dichloroacetic acid) have been employed in the present investigation. Andersen has reported[11] on the partition column chromatography of insulin and insulin derivatives in a solvent system composed of 2-butanol–0.01 *N* trichloroacetic acid. In contrast to the present work, Andersen used reverse phase chromatography where the organic phase was held on the column. Andersen found the technique very useful in the separation of the A and B chains of oxidized insulin and in the separation of phenylcarbamoyl derivatives of insulin. However, he apparently did not encounter the heterogeneity in the eluted insulin which is indicated in this report.

Experimental

Materials.—The insulin samples studied were all crystalline zinc insulin of high purity (23–27 units/mg.) and of bovine origin.[12] One sample (Lilly T-2842) had been recrystallized five times. For some experiments the insulin was converted to the hydrochloride before use. This was done by lyophilization of a 1.7% insulin solution in 0.1 *N* hydrochloric acid followed by lyophilization from water. The insulin hydrochloride was stored at −10° until used.

Commercial 2-butanol was purified by distillation through a 1 meter long, vacuum jacketed column packed with glass helices. The distillation was conducted at reflux ratios of thirty to one until the distillate had reached a nearly constant value of light absorption at 275 mμ and then the bulk of the distillate was collected at reflux ratios of three to one. In general 2-butanol having an optical density of 0.15 or less when read against distilled water in a 1 cm. cuvette was used in the partition column experiments.

The support for the aqueous phase was Hyflo Super-Cel[13] (Hyflo). The Hyflo (500 g.) was shaken with 2 *N* hydro-

(1) This work was supported in part by grants from Eli Lilly and Company and grant A-608C of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

(2) G. P. Hess and F. H. Carpenter, THIS JOURNAL, **74**, 4971 (1952).

(3) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 1358 (1941).

(4) L. C. Craig and D. Craig, "Techniques of Organic Chemistry," Vol. 3, ed. by A. Weissberger, Interscience Publishers, Inc., New York, N. Y., 1950, ch. 4.

(5) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **73**, 877 (1951); **74**, 3083 (1952).

(6) J. R. Weisiger, in "Organic Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1954, pp. 295–296.

(7) E. Hecker, "Verteilungsverfahren in Laboratorium," Verlag Chemie, GmbH., Weinheim, Germany, 1955.

(8) S. W. Mayer and E. R. Tompkins, THIS JOURNAL, **69**, 2866 (1947); E. Glueckauf, *Trans. Faraday Soc.*, **51**, 34 (1955).

(9) A theory of chromatography based on analogy with countercurrent distribution has recently been published by M. Verzele and F. Alderweireldt, *Bull. soc. chim. Belg.*, **64**, 579 (1955).

(10) R. R. Porter, *Biochem. J.*, **53**, 320 (1953).

(11) W. Andersen, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **29**, 49 (1954); *Acta Chem. Scand.*, **8**, 359 (1954); **8**, 1723 (1954).

(12) The authors wish to express their appreciation to Dr. O. K. Behrens of Eli Lilly and Co., Dr. E. E. Hays of Armour and Company and Dr. A. M. Fisher of the Connaught Medical Laboratories for providing generous samples of insulin.

(13) Johns-Manville Products Corporation.

chloric acid (3 l.) for 2 hours, collected on a filter and washed copiously with distilled water. The acid treatment followed by water washing on the filter was repeated. After the resulting Hyflo had been shaken twice for two hours in water (3 l.), it was suspended and shaken twice for two hours with 2-butanol (3 l.). The washed Hyflo was allowed to dry in air at room temperature for two weeks before use. Hyflo prepared in this manner was found to have a density of about 3.0.

Column Design.—Figure 1 shows a schematic diagram of the columns used. Such columns provided an adjustable but constant flow rate, did not introduce ultraviolet absorbing materials to the solvents, and could be completely dismantled for cleaning. The columns were constructed from commercially available burets.[14] A glass wool plug followed by a thin layer of asbestos fibers was tamped in place on the top of the conical, Teflon wedge to form a support for the column packing.

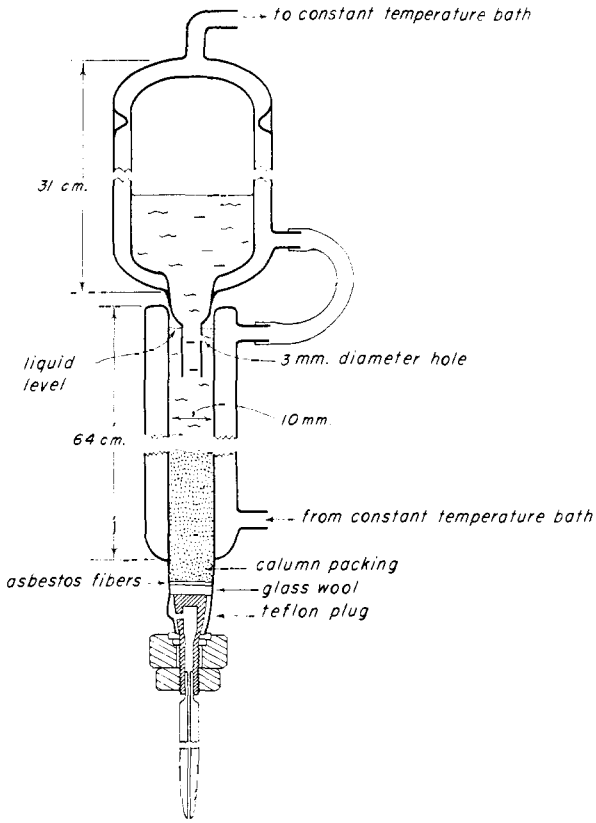

Fig. 1.—Diagram of the column apparatus used in the partition column chromatography of insulin.

Preparation of Column.—The solvent systems used were prepared by equilibrating 2-butanol with an equal volume of water containing various amounts of dichloroacetic acid (DCA) and hydrochloric acid. Equilibration was performed in a jacketed separatory funnel through which water at 24.5 ± 0.5° was circulated and the separated phases were maintained at the same temperature. The systems are designated according to their composition before equilibration.

A weighed amount of Hyflo (from which the volume could be calculated from the density) was thoroughly mixed with a known volume of equilibrated lower (aqueous) layer (generally 0.66 ml./g. of Hyflo) with precautions against heating or cooling. All of the upper layer that was to be used in developing the column was added to the aqueous Hyflo mixture and the resulting slurry was stirred for 1.5 hours at 24.5°. Most of the upper layer was decanted and the remaining slurry was poured into the water-jacketed column which was occasionally rotated back and forth along its vertical axis while the Hyflo was settling in order to remove entrained air bubbles.

Following packing, the excess solvent was removed from the column and the sample, dissolved in the moving phase, was added. Unless otherwise noted the sample was added in a concentration of 5 mg./ml. Care was exercised during these operations to prevent the surface of the column from becoming dry. In most instances a flow rate of 0.055 to 0.075 ml./sq. cm. cross section/min. was used.

The collection of fractions, which was started when the sample was added to the column, was performed by means of a photo-electric drop-counting collector.[15] The drop size was calibrated for each solvent and flow rate used. For 1 cm. (i.d.) columns fractions of approximately 1.6 ml. were collected. In order to minimize evaporation, the fractions were removed from the collector at 8 to 12 hour intervals and stored in an air-tight box which contained trays of the solvents in order to saturate the enclosed atmosphere.

The insulin was determined in the eluted fractions by measurement of the optical density reading at 275 mμ in a 1 cm. cuvette against distilled water as a blank. In order to ensure homogeneous solutions, 0.2 ml. of absolute ethanol per 1.6 ml. of moving phase was added before reading the tubes. Standard solutions of insulin containing 1 mg. per ml. gave optical density readings of 1.0 ± 0.03 for 1 cm. thick solutions. This value did not differ appreciably for the various insulin samples contained in the various solvents.

Theory

Partition Factor (K') and Distribution Constant (K).—The packed column is considered to be made up of a number (N) of "theoretical plates or tubes"[3] each of which contains a volume (v_m) of moving phase and a volume (v_s) of stationary phase analogous to the upper and lower layers in a CCD train, from which it follows that the total volume of moving phase held in the column (*hold-up volume*, V_H) and the total volume of stationary phase used in preparing the column (V_S) are defined by the relationships

$$V_H = N(v_m),\ V_S = N(v_s) \qquad (1)$$

The fraction (T_w) of the total amount of solute introduced on the column that occurs in the wth eluted volume of size v_m is defined by the Pascal distribution formula[6,7,16]

$$T_w = \frac{(N + w - 1)!}{w!\,(N - 1)!}\, p^N q^w \qquad (2)$$

where the first v_m eluted after the hold-up volume of the column (V_H) has come off is numbered zero (w_0) and subsequent volumes are numbered w_1, w_2, ... The values p and q represent the fractions of the total amount of solute present in a theoretical plate which occur in the moving and stationary phase, respectively, and are determined by the relations[17]

$$p = K'/(K' + 1) \qquad (3)$$

$$q = 1/(K' + 1) \qquad (4)$$

where K' is called the *partition factor* and is equal to the ratio of the total amount of solute in the moving phase to the total amount of solute in the stationary phase of a theoretical plate. The partition factor (K') is in turn related to the *distribution constant* (K), defined as the ratio of the concentration of solute in the moving phase to the

(14) Ultramax buret, Fischer and Porter Company, Hatboro, Penna.

(15) Technicon Chromatography Corp., N. Y., N. Y.

(16) W. Feller, "An Introduction to Probability Theory and Its Applications," Vol. 1, John Wiley and Sons, Inc., New York, N. Y., 1950, pp. 218, 31.

(17) S. Stene, *Arkiv Kemi, Mineral. O. Geo.*, **18A**, 1 (1944).

concentration in the stationary phase of a theoretical plate, by the formulas

$$K' = K(v_m)/(v_s) = K(V_H)/(V_S) \quad (5)$$

In a Pascal distribution where N is large (>100) the mean is closely approximated by Nq/p and the standard deviation by $(Nq/p^2)^{1/2}$ (ref. 6, 16). From the mean it follows that

$$w_{max} = Nq/p = N/K' \quad (6)$$

where w_{max} is the number of the eluted fraction of size v_m that contains the maximum concentration of solute. From equations 1 and 6 and from the fact that w_{max} is also closely approximated by

$$w_{max} = (V_E - V_H)/v_m \quad (7)$$

where V_E is called the *elution volume* and is equal to the total amount of eluate collected from the time the solute is introduced on the column up to and including the eluted fraction of maximum concentration, one can derive the following expressions for the *partition factor* and the *distribution constant*

$$K' = V_H/(V_E - V_H) \quad (8)$$

$$K = V_S/(V_E - V_H) \quad (9)$$

All of the values on the right-hand side of the above equations are measured directly in the course of an experiment with the exception of V_H. The latter can be measured indirectly from a knowledge of the geometry of the column and its measurable contents by

$$V_H = \pi r^2 h - (V_S + V_{sup}) \quad (10)$$

where r is the radius of the column, h is the height of the packed column and V_{sup} is the volume of the inert support used in preparing the column. The latter can be calculated from weight and density measurements. In most of the columns described in this paper, which had radii of 0.515 cm., 4 ml. of stationary phase (V_S) was used with 6 g. of Hyflo (V_{sup} = 2 ml.) to give columns with heights of about 25 cm. resulting in values for V_H of about 15 ml.

Number of "Theoretical Plates" on the Column (N).—Since the efficiency of a column in any particular separation will be a function of the number of "theoretical plates" describing its performance (N) (analogous to the number of tubes in a CCD train) and since this number may vary from column to column, it is advantageous to have a means of calculating N for each experiment. A convenient method for doing this is derived from the fact that the width of the elution band in volume units between the two points on the elution curve of concentration equal to one-half the maximun concentration (V_B) is related to the standard deviation as

$$V_B/v_m = 2.355(Nq/p^2)^{1/2} \quad (11)$$

Upon the substitution of equivalent expressions for v_m, p and q from equations 1, 3, 4 and 8 and solving for N one obtains

$$N = 5.55[(V_E)^2 - V_E V_H]/(V_B)^2 \quad (12)$$

Optimum Partition Factors.—In the use of partition columns for the separation of mixtures it is important to know the relationship that exists between the partition factors of closely related substances and the degree to which they may be separated on any particular column. The partition factor (K') is determined not only by the distribution constant (K) but also by the relative volumes of the two phases. It is generally possible to vary the volumes of the two phases as well as their composition in such a manner as to vary the partition factor over a wide range. Such changes in experimental conditions should be directed so as to give a partition factor which allows the maximum separation of closely related substances on any particular column.

Equations which are of aid in determining the optimum partition factor can be derived from considerations similar to those proposed by Weisiger for CCD.[6] Assume one has an equal mixture of components a and b with partition factors of K'_a and K_b. The partition factors for these two components can be related to each other as

$$K_b = \beta K'_a \quad (13)$$

where β is the ratio of the partition factors of the two components with the largest value assigned to component b. Since better than 99.7% of component a or b will be found in the number of eluted fractions of size v_m determined by the mean ± 3 standard deviations, one can consider the two components completely separated when S of the following equation is ≧ 1.

$$S = \frac{N/K'_a - N/\beta K'_a}{[(6N(K'_a + 1)/(K'_a)^2) + 6N(\beta K'_a + 1)/(\beta K'_a)^2]/2} \quad (14)$$

The value S is the ratio of the distance between the elution peaks of a and b to the sum of one-half the distance covered by each elution band. Values of S that are less than one can be used as an index to the degree of separation of the two components. Simplification of equation 14 yields the relationship[18]

$$S = N^{1/2}(\beta - 1)/3[\beta(K'_a + 1)^{1/2} + (\beta K'_a + 1)^{1/2}] \quad (15)$$

If one assumes a constant β-value and a constant number of theoretical plates on a column, it is possible to calculate the degree of separation (S) as a function of K'_a from equation 15. Figure 2 shows a calculation of this type where N is fixed at 400 and for β-values of 1.125, 1.250, 1.500 and 2.000. It can be seen from this example that as the value for K'_a decreases the degree of separation increases for all β-values. However, the rate of increase in S as K'_a decreases falls off rapidly at values of K'_a below 0.1. Therefore, in this example, little would be gained by working with K' values of less than 0.1 for the main component.

The shape of the curves in Fig. 2 indicates that as the K'_a value decreases the degree of separation (S) increases toward a limit value. As K'_a approaches zero, the terms $(K'_a + 1)^{1/2}$ and $(\beta K'_a + 1)^{1/2}$ of equation 15 both approach one as a limit. Upon substitution of these limit expressions in equation 15 one obtains

$$\lim_{K_a' \to 0} S = N^{1/2}(\beta - 1)/3(\beta + 1) \quad (16)$$

By use of equation 16 it is possible to calculate

(18) The S of this equation is equivalent to the $\theta S6$ of Weisiger who proposed a similar derivation but evidently made an algebraic error in its solution (ref. 6, p. 296).

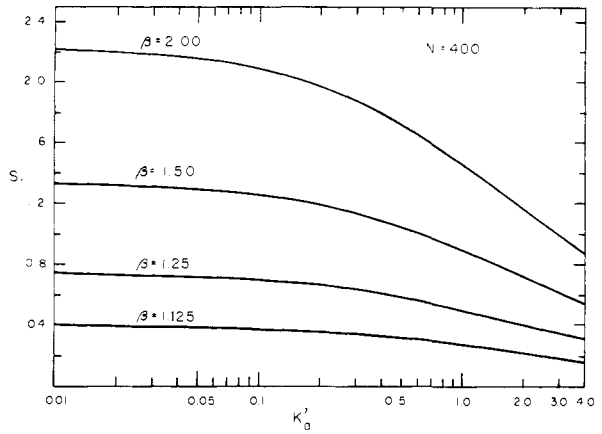

Fig. 2.—Plot of the degree of separation (S) of two components against the K'_a value (on a logarithm scale) of the component with the lowest partition factor for several β-values on a column represented by 400 theoretical plates.

rapidly the limits of separation that can be anticipated by changing the K' values and as a consequence one can reach a rational decision on an experimental approach.

Results and Discussion

Initial Experiments.—The solvent system of Harfenist and Craig[5] (2-butanol–1% DCA) proved unsatisfactory for partition columns as the insulin came off the column very shortly after the hold up volume (V_H). This result might be expected from the values calculated for the *partition factor* (K') on the basis of the value reported by Harfenist and Craig[5] for the distribution constant (K) and the volumes of stationary (V_S) and moving phase (V_H) in the column. The incorporation of 0.01 N hydrochloric acid into the aqueous layer resulted in a marked decrease in the value of the distribution constant as determined by the elution volume (V_E) of the material from a column. Figure 3a shows the results of such an experiment where the moving phase was 2-butanol–0.5% DCA in 0.01 N hydrochloric acid. These results would indicate the separation of the insulin into at least three components, one moving essentially with the solvent front and two other components with distribution constants of 0.15 (I) and 0.3 (II). With careful attention to experimental detail this result could be repeatedly duplicated. However, when material was isolated from one of the peaks and resubjected to chromatography under the same conditions, it gave an elution curve (Fig. 3b) very similar to that obtained on the original sample. This result indicated that the apparent separation shown in Fig. 3a was probably an artifact.

Figure 4 shows the results obtained in a series of experiments where the total amount of insulin put on the column was varied. With 7.5 mg. of insulin (Fig. 4a) most of the material came out in a nearly symmetrical peak with a K value of 0.14. Small amounts of material were found at the solvent front and at an elution volume corresponding to a K value of 0.28. As the amount of insulin put on the column was increased the amount of material appearing with K value of 0.28 increased while the

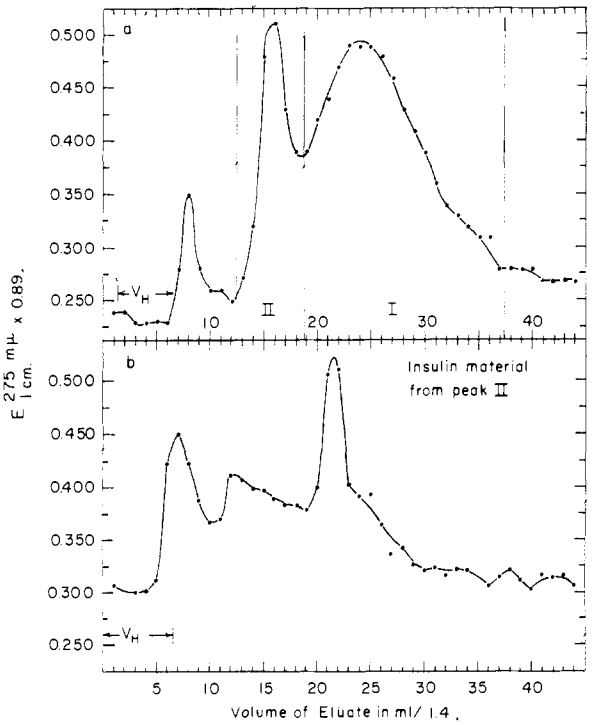

Fig. 3.—Optical density of fraction *vs.* volume of eluate for chromatography of insulin. Solvent was 2-butanol–0.5% DCA in 0.01 N hydrochloric acid, V_S = 4 ml., 6 g. Hyflo: (a) 10 mg. of zinc insulin (Lilly T2842) added in 1.5 ml.; (b) 3.3 mg. of material from fraction II of (a) put on in 1 ml.

amount of material moving with a K value of 0.14 remained fairly constant. At a load of 20 mg. of insulin (Fig. 4d), most of the material came out at an elution volume corresponding to a K value of about 0.28. In these experiments the volume in which the insulin had been added to the column was kept constant which resulted in a change in concentration of the added insulin as well as a change in the total amount added. In order to determine which of these variables was contributing to the anomaly, a series of experiments was performed in which the total amount of insulin was kept constant, but the concentration was varied. Figure 5 shows that varying the concentration of insulin put on the column, while keeping the total amount constant, had no effect. Therefore, the increase in the amount of material that appeared in the elution peak corresponding to a K value of 0.28 as the load was increased over 7.5 mg./column must be attributed to the amount of insulin put on the column rather than the concentration in which it was put on the column. It should be noted that the small amount of material eluted shortly after the hold-up volume is probably a transformation product of insulin (perhaps *fibril* insulin[19]) which may be formed in part in the solvent systems.

Experiments were also conducted on the effect of varying the hydrochloric acid and the DCA concentrations on the value of the distribution constant of insulin as determined by 49 transfer CCD

(19) D. F. Waugh, THIS JOURNAL, **66**, 663 (1944); **68**, 247 (1946); **70**, 1850 (1948).

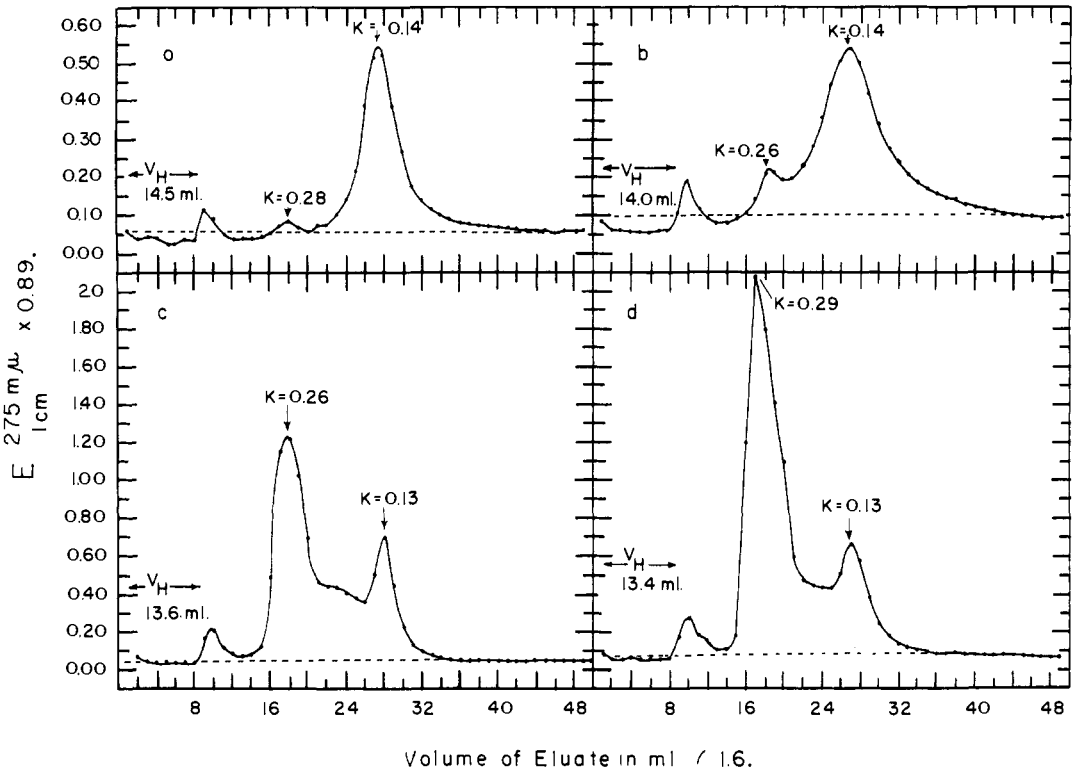

Fig. 4.—The effect of amount of insulin put on column on the shape of the elution curve. Solvent was 2-butanol–0.5% DCA in 0.01 *N* hydrochloric acid; *V*s = 4 ml., 6 g. of Hyflo. Variable amounts of crystalline zinc insulin (Connaught 885) put on column in 1.5 ml. of moving phase: (a) 7.5 mg.; (b) 11.25 mg.; (c) 15.0 mg.; (d) 20.0 mg.

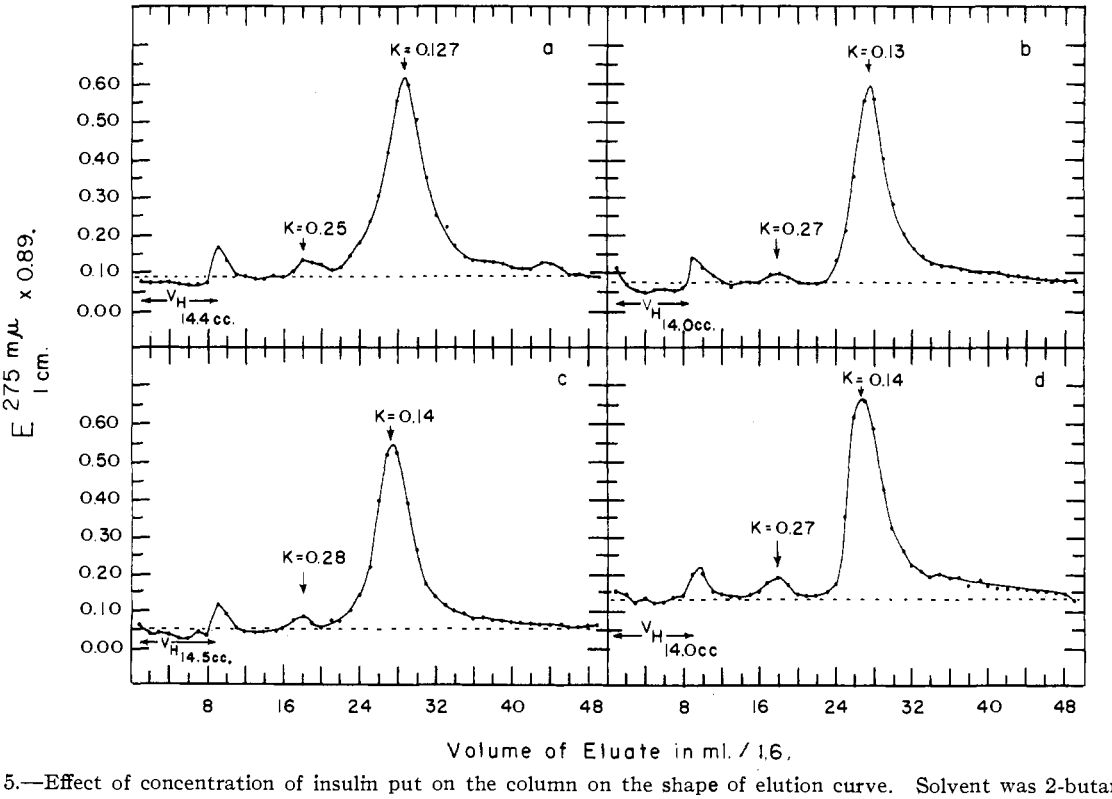

Fig. 5.—Effect of concentration of insulin put on the column on the shape of elution curve. Solvent was 2-butanol–0.5% DCA in 0.01 *N* hydrochloric acid. *V*s = 4 ml., 6 g. of Hyflo. 7.5 mg. of zinc insulin (Connaught 885) put on the column in varying amounts of moving phase to give concentrations of: (a) 15 mg./ml.; (b) 10 mg./ml.; (c) 7.5 mg./ml.; (d) 2.5 mg./ml.

experiments. The results (Fig. 6a) showed the very marked influence of small concentrations of hydrochloric acid on the value of the distribution constant between 2-butanol and 0.5% DCA. The value of the distribution constant dropped abruptly from about 0.37 in 2-butanol—0.5% DCA to about 0.10 in 2-butanol—0.5% DCA in 0.01 N hydrochloric acid. Further increases in hydrochloric acid concentration of the system had little effect on the values of K. The effect caused by changes in the DCA concentration (Fig. 6b) were relatively minor as compared to the abrupt change observed in going from solvents without hydrochloric acid to those containing 0.01 N hydrochloric acid.

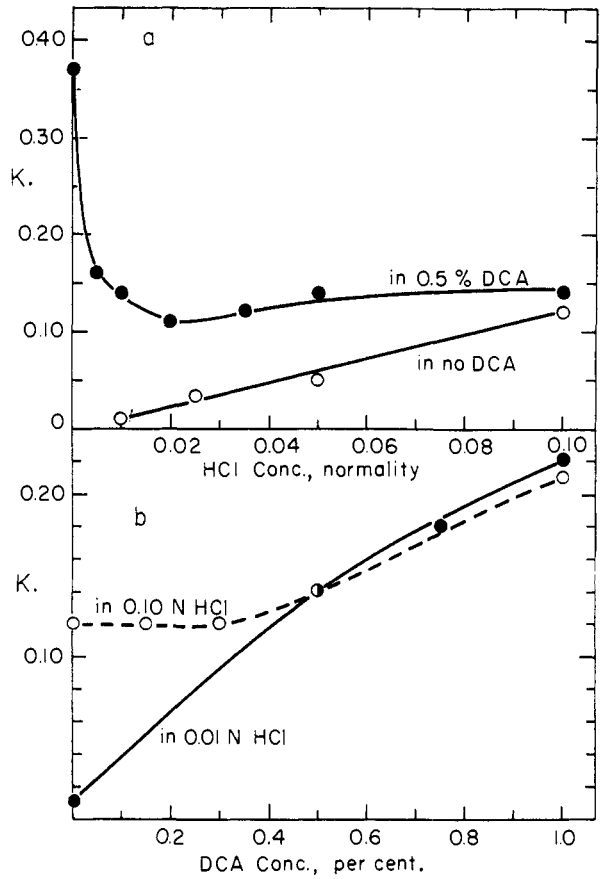

Fig. 6.—Distribution constant of zinc insulin between 2-butanol and water containing various amounts of DCA and hydrochloric acid as determined by 49-transfer CCD experiments: (a) K as a function of hydrochloric acid concentration in the presence and absence of 0.5% DCA; (b) K as a function of DCA concentration in 0.01 N and 0.1 N hydrochloric acid.

In further investigations, the composition of the aqueous and organic layers at various concentrations of hydrochloric acid was studied. The results indicated that the hydrochloric acid was contained largely in the aqueous phase while the DCA (in the presence of small amounts of hydrochloric acid in the system) was contained almost entirely in the organic phase. The distribution constant of the hydrochloric acid between the two phases remained practically constant (K = 0.33) with increasing hydrochloric acid concentration.

The information obtained from studies on the factors affecting the K value of the insulin is of aid in explaining the anomaly encountered in the partition column chromatography of insulin as depicted in Figs. 3 and 4. It would appear that in solvent systems containing hydrochloric acid and DCA, the more highly dissociated hydrochloric acid bears the primary influence in determining how the insulin will distribute between the two phases. When small amounts of isoelectric insulin are placed on the column, the insulin is largely converted to the hydrochloride which partitions between the two layers with a relatively small distribution constant. However, upon the addition of increasing amounts of isoelectric insulin to the column, one saturates the hydrochloric acid of the system and the DCA salt begins to form. The latter, which has a larger distribution constant than the hydrochloride, moves ahead down the column. According to this explanation the material moving with a K value of 0.14 was partitioning largely as the hydrochloride, while the material moving with a K value of 0.28 was partitioning largely as the DCA salt, and the material in between the two peaks was partitioning as various mixtures of the two forms.

Chromatography of Insulin Hydrochloride between 2-Butanol and 0.5% DCA in 0.1 N Hydrochloric Acid.—If the cause of the anomalies encountered in the initial experiments was the result of the neutralization of the small amount of hydrochloric acid in the system by the addition of insulin in its isoelectric form, one should be able to avoid this by increasing the hydrochloric acid in the system or by introducing the insulin as its hydrochloride.

In a series of experiments in which the hydrochloric acid concentration of the lower layer before equilibration was varied from 0.01 to 0.2 N, it was observed that at concentrations of hydrochloric acid of 0.1 N or greater the amount of insulin put on the column in its isoelectric form could be varied several fold without markedly changing the shape of the elution curve. If, in addition, the insulin was put on the column as the hydrochloride, the amount added could be varied by at least sevenfold without changing the nature of the elution curve appreciably (see Fig. 7). The results of these experiments are in sharp contrast to those of Fig. 4 where noticeable effects were observed over much smaller ranges of material. Results such as those shown in Fig. 7 suggest that under these conditions the column is functioning as a true partition column in which the location of the material in the eluate fractions is a function of its partition factor (K'). Further evidence for this conclusion was obtained from a study of the effect of varying the amount of stationary phase on the position of the elution peaks. Figure 8 shows the results of a series of experiments where the volumes of the stationary phase used in the preparation of the column were varied over a 3-fold range. These changes in stationary phase were reflected in the values for the partition factor (K') as calculated from the elution volume for the main component. Partition factor values of 0.73, 0.41 and 0.20 were

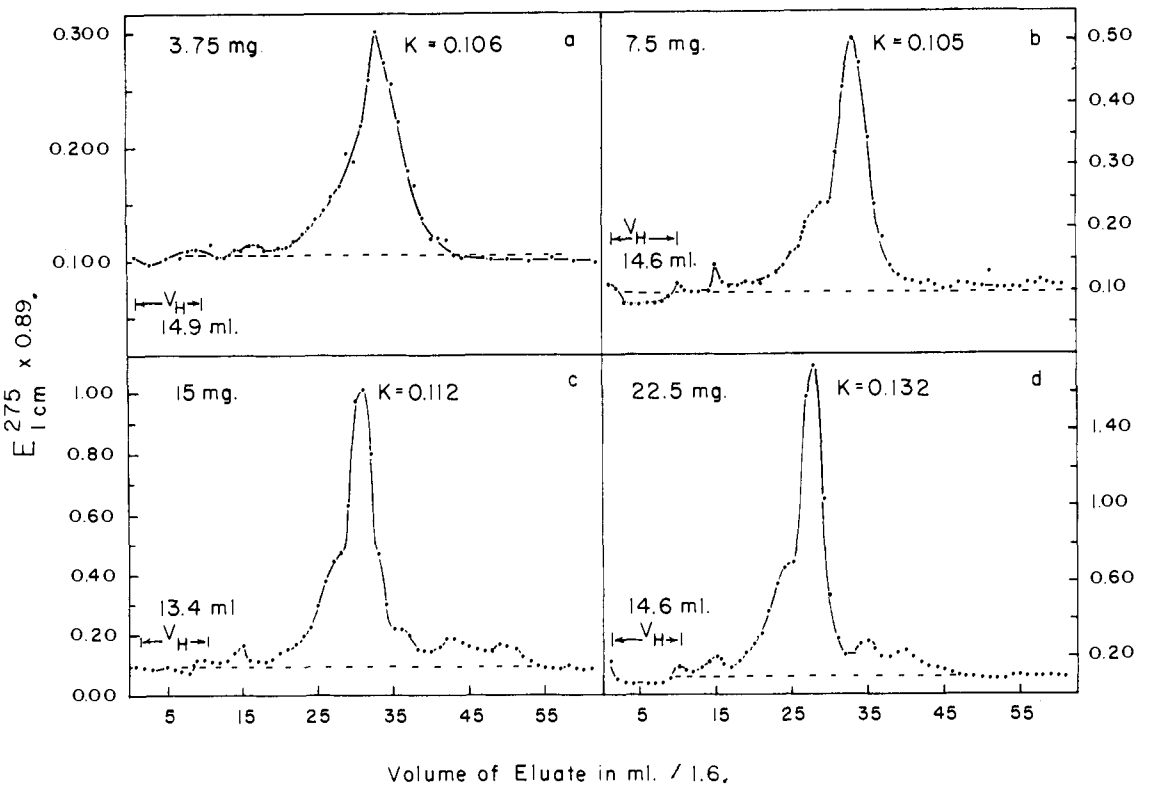

Fig. 7.—Partition chromatography of various amounts of insulin hydrochloride. Solvent was 2-butanol–0.5% DCA in 0.10 *N* hydrochloric acid. *V*s = 4 ml., 6 g. of Hyflo. Various amounts of insulin (Lilly 535664) as the hydrochloride were put on the column in a volume of 1.5 ml.: (a) 3.75 mg., 79% recovery, calcd. $N = 138$; (b) 7.5 mg., 68% recovery, calcd. $N = 226$; (c) 15 mg., 78% recovery, calcd. $N = 288$; (d) 22.5 mg., 81% recovery, calcd. $N = 130$.

found for the column with 2, 4 and 6 ml. of stationary phase per 6 g. of Hyflo. Thus, changes in the K' values are very close to those that would be predicted on the basis of the changes in the volumes of stationary phase on the column. At the same time the distribution constant (K), as calculated from the elution volume of the main component, remained practically unchanged.

Since the above evidence indicated that the columns were functioning as true partition columns, one should be able to attach some significance to the shape of the elution curves presented in Figs. 7 and 8. Concentrating for the moment on the main elution band, it is obvious from inspection that the band does not have the symmetrical shape expected for one component. It would appear to be made up of a main component with a K value of about 0.11 and a smaller, faster moving component which appears as a shoulder on the main band. In this respect the results from the partition column are similar to the results of Harfenist and Craig on the CCD of insulin.[5] That the shoulder on the main peak was real rather than an artifact, was demonstrated in a series of experiments wherein the material was isolated from various areas of the band and subjected to rechromatography. Figure 9a shows an elution curve for a sample of crystalline insulin in which the main elution band was divided into three fractions. The material was isolated from these fractions[20] and then separately subjected to chromatography. Fraction I from the right-hand side of the elution band gave a nearly symmetrical curve with a calculated K value of 0.114 (see Fig. 9b). Although Fraction I gave rise to an elution curve that was a much closer approximation to a theoretical elution curve for one component than that obtained on the original insulin sample, the observed result was still not in perfect agreement with a theoretical curve in that the tails of the experimental curve were somewhat too broad.

When fraction II, which should have contained material from the shoulder and the main peak in about equal amounts, was rechromatographed, it gave rise to a broad elution band with two distinct peaks corresponding to K values of 0.125 and 0.108 (see Fig. 9c). When fraction III, isolated from the shoulder on the left of the main elution band, was rechromatographed, it gave rise to a band with an elution volume corresponding to a K value of about 0.174 (see Fig. 9d). Thus each of the fractions gave rise to an elution curve on rechromatography that was qualitatively in agreement with the result that one would predict for these fractions on the basis of their origin.

The material which was eluted shortly after the

(20) The solvent in the tubes of the fraction was pooled, diluted with an equal volume of 0.1 *N* hydrochloric acid and extracted three times with an equal volume of ether (U.S.P.). The aqueous layer was then lyophilized. The residue was redissolved in a small amount of water and relyophilized to give a white amorphous powder.

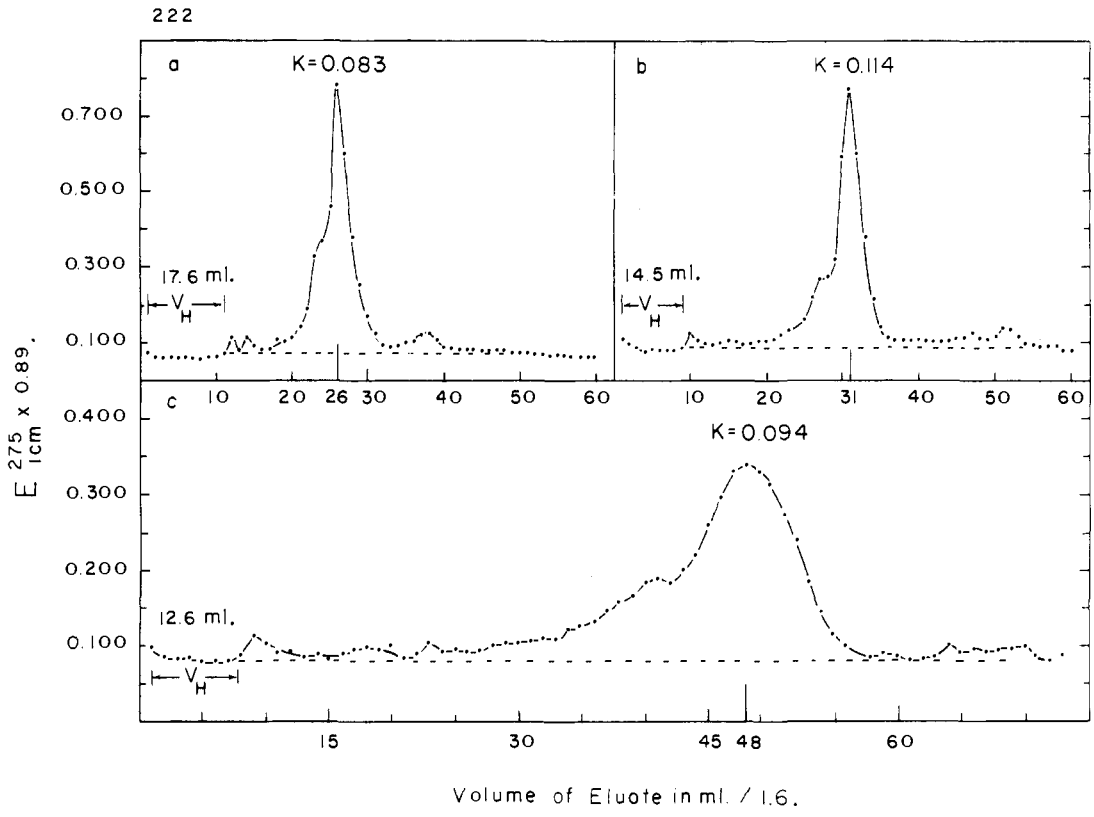

Fig. 8.—Partition chromatography of insulin hydrochloride as a function of the volume of stationary phase used in preparing column. Solvent was 2-butanol–0.5% DCA in 0.1 N hydrochloric acid. 6 g. of Hyflo. 7.5 mg. of insulin (Lilly 535664) as the hydrochloride put on column in a volume of 1.5 ml.: (a) V_S = 2 ml., 85% recovery, calcd. N = 276; (b) V_S = 4 ml., 88% recovery, calcd. N = 337; (c) V_S = 6 ml., 87% recovery, calcd. N = 160.

hold-up volume and the materials eluted after the main band (see Figs. 7 and 8) were relatively small and also variable in amount from experiment to experiment. Because of this it is highly probable that these minor components are transformation products of insulin which may have been formed in part during the process of converting the insulin to its hydrochloride or even during the chromatography procedure.

Recovery of Material from the Columns.—The total recovery of the ultraviolet light absorbing materials from the column is given in the legends to the figures. The recovery is calculated on the total amount of ultraviolet absorbing material that was eluted after the hold-up volume and after being corrected for the base line, represented by the dotted line. The recovery of ultraviolet absorbing material was rarely as high as 90% and averaged around 80%. The reasons for these low recoveries is obscure. When the insulin was dissolved in either the lower or upper layer and run on a column without any stationary phase, better than 95% of the material was eluted from the column shortly after the hold-up volume. Thus, irreversible adsorption from either phase onto the Hyflo appears to be eliminated. When the same column was used for several chromatographic experiments or when material was isolated from an elution curve and rechromatographed (see Fig. 9), the recovery was still not improved appreciably.

Recoveries of around 80% are sufficient to consider the use of these columns for the isolation of insulin from biological materials. However, the low recoveries restrict observations on the homogeneity of the material to that fraction of the sample which is obtained in the eluate. Remarks on the homogeneity of the original sample would have to take into consideration the fact that 15 to 20% of the sample was not recovered.

Considerations on the Performance of the Columns.—In most of the experiments reported here, in which 0.66 ml. of stationary phase per 1.0 g. of Hyflo was used, the partition factor (K') for the major component in the elution peak was about 0.4. The material in the shoulder had a slightly higher K' value which was estimated to be about 0.5. Thus the ratio of the partition factors gives a β-value of about 1.25. According to the theoretical development, one would not expect to separate these two components completely in a column of less than 730 theoretical plates. The number of theoretical plates (N) actually calculated for the various experiments from the width of the elution band of the main peak at $^1/_2$ the maximum concentration (equation 12) is given in the legend to the figures. This value which varied somewhat from experiment to experiment was in the order of 200 to 300 theoretical plates for a column made up of 4 ml. of stationary phase and 6 g. of Hyflo. The value is considerably short of the number of theoretical plates needed for complete separation of components. Better separation of the components

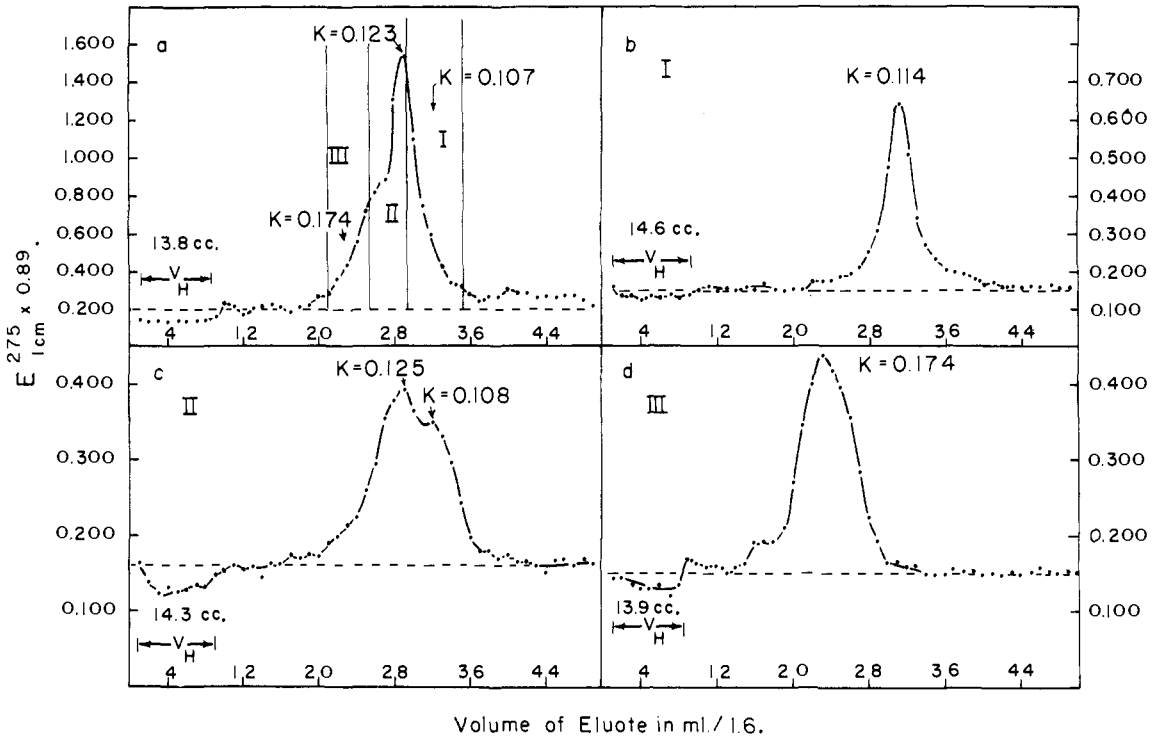

Fig. 9.—Partition chromatography of an insulin hydrochloride sample and of fractions obtained from the original sample. Solvent was 2-butanol–0.5% DCA in 0.1 N hydrochloric acid. V_S = 4 ml., 6 g. of Hyflo.: (a) 20 mg. of insulin (Lilly 535664) as hydrochloride put on column in 1.5 ml., 78% recovery, calcd. N = 253; (b) 5 mg. of fraction I put on column in 0.5 ml., 88% recovery, calcd. N = 419; (c) 5 mg. of fraction II put on column in 0.5 ml., 80% recovery; (d) 5 mg. of fraction III put on column in 0.5 ml., 77% recovery, calcd. N = 42.

might be expected by lowering the partition factor to a value closer to 0.1. One way in which the partition factor may be lowered is to increase the volume of stationary phase on the column. Figure 8c shows the results obtained when the ratio of stationary phase to support was increased to 1 ml./g. This resulted in a drop of partition factor for the main component to a value of about 0.2 while the β-value remained at 1.25. Unfortunately the number of plates on the column dropped to 160 and as a result the separation of components was not appreciably better than that realized at the higher K' value.

Further lowering of the partition factor by further increases in the amount of lower layer is prohibited by the fact that there is a limit to the amount of stationary phase that can be held immobile by the inert support. Broad and variable elution curves were obtained in occasional experiments in which a ratio of 1 ml./g. and in almost all experiments in which a larger ratio of stationary phase to support was used. These results indicated that the limit of stationary phase that could be held immobile by the Hyflo, as it was prepared for these experiments, was about 1 ml./g. and that it was advisable to use a somewhat lower ratio (about 0.66 ml./g.) in order to achieve reasonable reproduction of results.

Further investigations in which a number of insulin samples have been subjected to partition column chromatography in a variety of solvents and conditions as well as the biological activity of the isolated components studied will form the subject of a future report.

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