ELUTION AND DISPLACEMENT ANALYSIS OF INSULIN AND ADRENOCORTICOTROPIC PEPTIDES ON PRE-TREATED CARBON

by

JERKER PORATH* AND CHOH HAO LI

Department of Biochemistry (Hormone Research), University of California, Berkeley, Calif. (U.S.A.)

Displacement analysis on charcoal[1,2] has previously been demonstrated to be useful for the separation of fatty acids[3], oligosaccharides[4], amino acids[5], peptides[6], and partial hydrolysates of proteins[7,8]. The present paper presents a further development of the method, applied to studies on insulin, and to the purification of adrenocorticotropic (ACTH) peptides.

THEORETICAL

One of the necessary conditions for the success of the carrier displacement method[2] is that the adsorption isotherms of the components in the mixture should have the same or almost the same shape as those of the carriers. In a recent paper, HAGDAHL, WILLIAMS AND TISELIUS[5] have shown that aromatic amino acids and small peptides form a group with more sharply curved isotherms, very different from substances consisting of uncharged molecules. Further, results from the application of carrier-displacement to a highly purified preparation of bacitracin[6] indicate that the main component, a polypeptide with a molecular weight of 1400–1500, must also have a sharply curved isotherm, presenting more evidence in support of the view that the sharp curvature is probably characteristic for all polypeptide isotherms.

In those cases where the substance to be purified is the main component of the mixture, a suitable concentration range can often be chosen in which the complications due to the crossing of the isotherms are minimized; but so far no homologous series of compounds has been found which could be used as a carrier to enable satisfactory separation of one component from another, or for enrichment of the active fraction, in a mixture where no one component dominates.

A slow rate of attainment of adsorption and tendency to irreversibility of adsorption represent the chief difficulties which arise in connection with the chromatography of

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* Permanent address: Biokemiska Institutionen, Uppsala, Sweden. Appreciation is expressed to Wenner-Grenska Samfundet, Stockholm, and the Swedish Medical Research Council for grants.

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polypeptides on charcoal. Desorption is made impossible because both of the above phenomena are caused, at least in part, by the simultaneous anchoring of the large molecules at too many points on the surface of the adsorbent. It is therefore necessary to reduce the number and/or the strength of the adsorption centers involved in the attachment of the molecules to the surface of the charcoal. This can be accomplished by modification of the adsorbent and/or the solvent.

The tendency to irreversible adsorption appears to be greatly reduced by mixing the charcoal with some inert or weak adsorbent such as diatomaceous earth. A selective elimination of surface active centers, however, is obtained by pretreating the adsorbent with a substance (saturator) which itself will be strongly adsorbed. This technique, which was introduced by TISELIUS and collaborators[9] blocks especially the strongest adsorption centers. The charcoal modified in this manner is a much weaker adsorbent exhibiting new adsorption characteristics. In extreme cases, no adsorption at all occurs. A study of the influence of pretreatment on charcoal for the separation of low molecular compounds has been reported by HAGDAHL, WILLIAMS AND TISELIUS[5], but it was not shown how far charcoal has to be saturated in order to make chromatography of high molecular compounds possible.

Strongly charged molecules, whether positive or negative, are adsorbed comparatively weakly. We have found that the charge exercises a very marked influence in the case of aromatic amines, which are strongly adsorbed in basic and neutral solutions, but only weakly so in acid media[10]. The same has been found to hold true for amino acids and polypeptides. It is therefore possible to increase or decrease the adsorption of such compounds by changing the pH.

It is considerably easier to develop suitable separation procedures when a biologically active substance is to be separated from a mixture of chemically similar but inactive components, since in that case adsorption and elution can be followed by bioassay. In the case of ACTH preparations we have thus found that the active substance remains on the charcoal if this adsorbent is not pretreated with a saturator. Moreover, at a low concentration of a strong saturator, such as *n*-decanol, the active substance cannot be eluted, and is desorbed only incompletely by a strong displacer. At higher concentrations of the saturator, the recovery of the active substance by displacement will be enhanced, and will finally be quantitative. At an even higher concentration of the saturator, not far from the point of saturation concentration where practically complete recovery is obtained, activity will be found in the elution part of the effluent. The limit of concentration of the saturator at which elution of the active substance occurs in this manner is determined by the kind of charcoal used, the ratio of charcoal/diatomaceous earth, the concentration of substance, etc., all factors which can easily be controlled. This limit will be called the *critical saturation point.*

For the separation of biologically active peptides, chromatography ought to be performed on charcoal saturated as near as possible to the critical saturation point* for elution. Under such conditions, the tendency to irreversible adsorption decreases rapidly when the saturation approaches the limit; it then becomes possible, as discussed above, to obtain a quantitative yield of the adsorbed substances. Furthermore, the

* The simplest way of controlling the degree of saturation is to perform the operation in the column, thoroughly washing the adsorbent with the saturator solution. The critical saturation point corresponds to the certain concentration of the saturator in the solvent at which the active component begins to appear in the eluate.

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amount of easily eluted peptides increases. In a series of experiments with progressively increasing saturation, where the analysis is performed in two steps—elution and displacement—the amount of substance which can be eluted increases at the expense of the amount of displaceable material. On the assumption that the active component in the peptide mixture is adsorbed more strongly onto the charcoal, when a saturation is chosen which is slightly below the critical saturation point of the active substance, the peptide mixture separates into two fractions. One of them, the eluate, contains practically all the substances adsorbed more weakly than the active component. The fraction which remains on the charcoal contains the active substance as well as those substances which are more strongly adsorbed. Most of the substances in the second fraction can be displaced from the charcoal with a strong displacer.

The next step in the enrichment of the active component(s) consists in the separation of the active substance from other contaminating inactive substances in the displaced material. Attempts to accomplish this latter separation by choosing a weaker displacer or lower displacer concentrations have not been successful. Two other means have been chosen instead: (a) *sectional displacement procedure*; i.e., taking apart sections of the column after the elution procedure and displacing the adsorbed material from each section separately, and (b) *rechromatography* on columns of different degrees of saturation.

The capacity for adsorption of the active component is small on charcoal which is saturated just below the critical saturation point for elution of this component. While the more strongly adsorbed substances will be retained in a region near the top of the column, the active substance will penetrate a larger volume of adsorbent and will be found in lower sections of the column. Since the fractions are thus localized, the procedure of sectional displacement is particularly advantageous since components of each adsorbed section can be displaced or eluted separately.

By submitting the active fraction of a previous experiment to rechromatography, using in this case a concentration of the saturator on the other side of the limit for elution of the active substance, it is possible to achieve a further separation. If in the first case the activity is located in the displaced fraction, after rechromatography under such conditions it is found in the eluate.

EXPERIMENTAL

Apparatus

The chromatographic column and the solvent container as connected in actual operation are shown in Fig. 1a, and the detail of their construction in Fig. 1b. The container consists of an outer cylinder, *A*, and a lid, *C*, made of lucite, an inner reservoir, *B*, of glass, and a metal base, *F*, with a plug, *G*, of Teflon, the latter to prevent contact between the solutions and the metal. The container is transparent so that the level of the solution can be watched, which greatly facilitates the procedure. The outer cylinder is screwed into the metal base which in turn is joined to the upper section of the column. The reservoir is a wide thick-walled glass tube, one end of which is drawn out to a narrow channel ending in a ground ball-joint. This ball-joint fits into a concavity in the Teflon plug, *G*, connecting the channel in the plug to the channel in the lower part of the glass container. Then the lid, *C*, is screwed onto the outer cylinder, and the reservoir fixed in a central position. The lid has two openings, one, *E*, at the top for convenient filling, and a side channel, *D*, which connects with an air pressure line.

The column is built up of units of coupled sections, according to the design of HAGDAHL[11], ending in an end piece, *O*, with a drop tip, *P*. All sections and connective parts are made of Teflon (for example *I* in Fig. 1b) encased in stainless steel; the latter material reinforces the column against breakage and strengthens the threads at the connecting points[12]. This construction fulfils the dual purpose of avoiding contamination of the solutions by the metal chlorides which would be formed by the corrosive effect of HCl on metals, and at the same time, of permitting easy filling.

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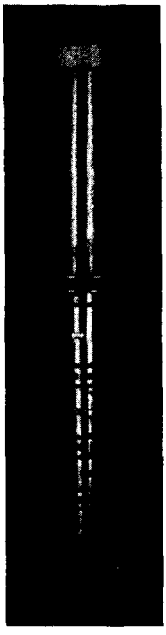
Each section is fitted with a perforated disk, K, inserted from above and pushed to the bottom, which is covered with a small piece of filter paper, L_2, before the column is packed. The adsorbent suspended in water is poured in and packed under suction[11].



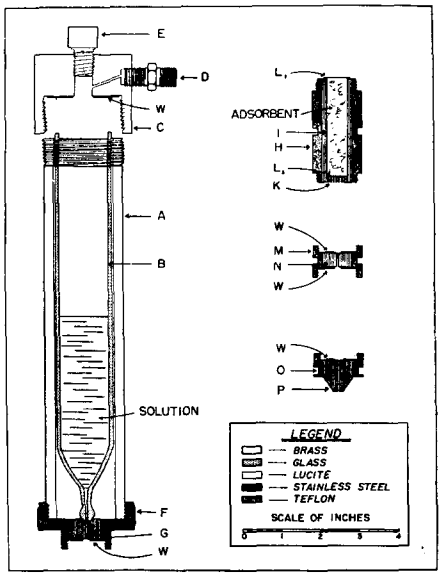


Fig. 1a. A photograph of the chromatographic column for elution and displacement analysis.
Fig. 1b. Construction of the chromatographic column. Solvent container: A, outer cylinder; B, inner container; C, lid; D, connection to air pressure reservoir; E, screw to the filling channel; F, metal base, G, Teflon plug; W, washers. Column section: H, stainless steel support (with indentions for wrench); I, Teflon tube; K, perforated Teflon disc; L_1, L_2, filter papers. Connective piece: M, metal support; N, Teflon plug; W, washers. End piece: O, metal support; P, Teflon plug, W, washer.

Adsorbent

A mixture of nine parts of Hyflo Supercel in one part of Darco G-60 was employed as the adsorbent, since previous experience with it has been very satisfactory[8]. Before use both the Hyflo Supercel and the charcoal are very thoroughly washed with concentrated hydrochloric acid followed by distilled water, and oven-dried at 100° C. A number of experiments using other proportions of charcoal to Hyflo Supercel indicated that progressively higher saturation of the charcoal is needed as the percentage of charcoal increases. Hyflo Supercel adsorbs high molecular peptides, especially from neutral and basic solutions, but under the conditions here described this adsorption is negligible compared with that occurring on the charcoal.

Solvent

Equal volumes of ethanol and 0.2 N hydrochloric acid are mixed and used as the solvent. In it are dissolved octanol or decanol in concentrations suitable for saturation of the charcoal. The higher alcohols are washed with 20% sulfuric acid solution, followed by washing with a 10% sodium hydroxide solution and finally distilled *in vacuo*.

Analytical methods

The nitrogen was estimated according to the Kjeldahl procedure and the ninhydrin colour by the method of MOORE AND STEIN[13]. Ultraviolet absorption was measured in a Beckman spectrophotometer at 275 mμ. The ACTH potency of the fractions was determined by the ascorbic acid depletion method[14] using the U.S.P. Standard as reference.

Materials

The ACTH concentrates were prepared from fresh whole sheep pituitary glands by the procedure

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previously described[15,16]. Beef insulin, five times crystallized (Lot T-2842) was supplied through the kindness of Dr. O. K. BEHRENS of the Eli Lilly Laboratories: Zn, 0.43%, N, 15.4%; biological activity, 26 units per mg.

RESULTS

Studies with insulin

Fig. 2 presents the adsorption capacity of the absorbent as a function of the degree of saturation by decanol, when insulin was used. The data was obtained by the following procedure: A column, consisting of one 25 ml section, was washed with about 100 ml of the solution. 10 mg of crystalline insulin was dissolved in 2 ml of the same solution, The eluate was collected and analysed for nitrogen. It may be seen from Fig. 2 that the insulin is completely adsorbed into the charcoal when the basic solvent is used. If the adsorbent has been previously saturated by 3%* decanol solution, the hormone is not adsorbed and can be completely recovered in the eluate. Thus it is possible to perform an elution analysis of insulin on pretreated charcoal with a decanol solution of 3% or higher.

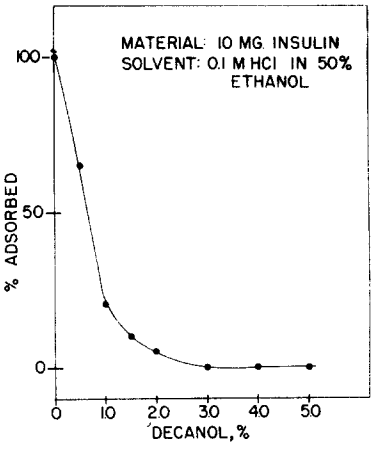

Fig. 2. Adsorption of insulin on carbon as a function of the degree of saturation by decanol.

A column of 2–25 ml sections was used; after the adsorbent had been placed in equilibrium with the 3% decanol solution, 10 mg of insulin in 2 ml of the same solution were introduced. Elution was performed under a pressure of 15 lbs. per sq. in. The eluant was collected in 3.2 ml fractions with an automatic fraction collector; after 23 fractions had been collected, a saturated decanol solution was introduced as the displacing agent. Each fraction was then read in a Beckman spectrophotometer at 275 mμ. It is evident from Fig. 3 that there was only one elution peak and no indication of strongly adsorbed material, which, if it were present, would be expected to be displaced out by saturated decanol solution. Nitrogen determination of the whole peak revealed a complete recovery of the protein hormone initially applied into the column.

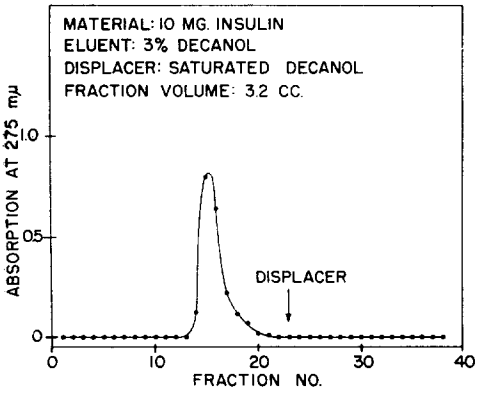

Fig. 3. Elution analysis of insulin on pretreated carbon with 3% decanol.

Since insulin can be adsorbed totally onto charcoal when the adsorbent has been treated with the basic solvent (0.1 *M* HCl in 50% ethanol), and since no adsorption is observed if the adsorbent is saturated with decanol, displacement analysis of the hormone on charcoal using decanol as the displacer would be of interest. Fig. 4 gives the results of a typical experiment. It is to be noted that no insulin appeared in the effluent before the dis-

* All percentages are expressed throughout as per cent by volume.

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placer was applied and that the hormone was displaced out as a single peak by decanol.

The hormone from a number of displacement analysis experiments was recovered after removal of the alcohol by ether and assayed by the mouse-convulsion procedure*. It was found that the hormonal potency is not impaired by adsorption and displacement manipulations. The original sample of insulin possessed an activity of 26 units per mg whereas the potency of the recovered material was 23 units per mg.

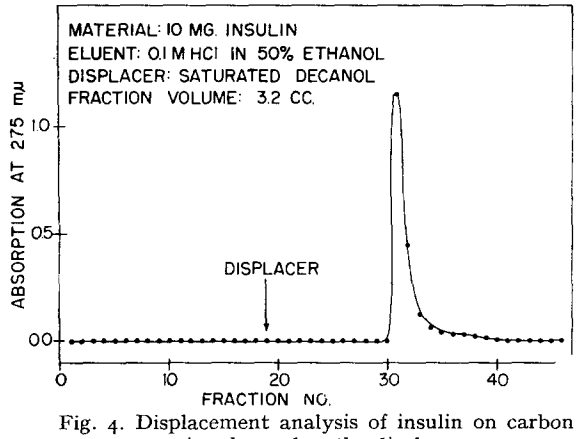

Fig. 4. Displacement analysis of insulin on carbon using decanol as the displacer.

Studies with ACTH peptides

In all experiments a ratio of amount of material in mg to volume of the section in ml between 0.5 and 1.25 was chosen. The material was dissolved in a volume of the saturator solution so that this solution contained 50–150 U.S.P. units of activity per ml.

Two series of experiments were performed. In one set, octanol or mixtures of octanol and decanol were used; in the other, various concentrations of decanol were employed. In the first series, with the decanol kept constant at 0.1%, the critical saturation point corresponded to a concentration of octanol between 0.5% and 0.7% (Table I). In the second series, 0.4% decanol gave an adsorbent saturated below the critical saturation point, whereas 0.6% decanol saturated the charcoal slightly above this point (Table II).

A column, built up of sections with the following volumes: 14.5, 14, 6.5, 5, 3.5, and 3.5 ml, was washed with a large volume (circa 200 ml) of the solvent containing 0.5% octanol and 0.1% decanol. 45 mg of an ACTH peptide preparation was dissolved in 20 ml of the same solution and pressed into the column. A pressure of 20 lbs per sq. in. gave a suitable flow rate (10–12 ml/h). Elution was performed with 75 ml of the same

TABLE I

NITROGEN AND ACTIVITY DISTRIBUTION OF FRACTION OBTAINED FROM CHARCOAL COLUMN USING OCTANOL AND OCTANOL-DECANOL MIXTURES AS SATURATORS

Concentration of saturators	*Nitrogen distribution*		*Location of activity*	
	Eluate	*Displaced fraction*	*Eluate*	*Displaced fraction*
%	%	%		
0.5% C_8*	57	33	—	+++
0.5% C_8 + 0.1% C_{10}**	65	22	—	+++
0.7% C_8 + 0.1% C_{10}	89	7	++	+
1.0% C_8 + 0.1% C_{10}	90	10	++	—

* abbreviation for *n*-octanol.
** abbreviation for *n*-decanol.

* The assay was carried out at the Eli Lilly Laboratories through the kindness of Dr. O. K. BEHRENS.

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TABLE II

NITROGEN AND ACTIVITY DISTRIBUTION OF FRACTIONS OBTAINED FROM CHARCOAL COLUMN USING *n*-DECANOL AS SATURATOR

% *n*-decanol	*Nitrogen distribution*		*Activity in USP units/mg*	
	Eluate	*Displaced fraction*	*Eluate*	*Displaced fraction*
	%	%		
0.1	48	11	< 1	< 1
0.4	70	21	< 6	125
0.6	82	9	10	150
0.8	87	13	30	12

solution, and was then followed by displacement with 75 ml of a saturated decanol solution. Data of nitrogen distribution and results of biological assays are compiled in Table III; the distribution of ninhydrin-reacting material plotted against effluent volume is presented in Fig. 5. It may be seen that the main elution fraction (E_1) contains 55% of the total nitrogen and has a potency of less than 6 U.S.P. units per mg, whereas the displaced fraction (D_1) possesses practically all the ACTH activity.

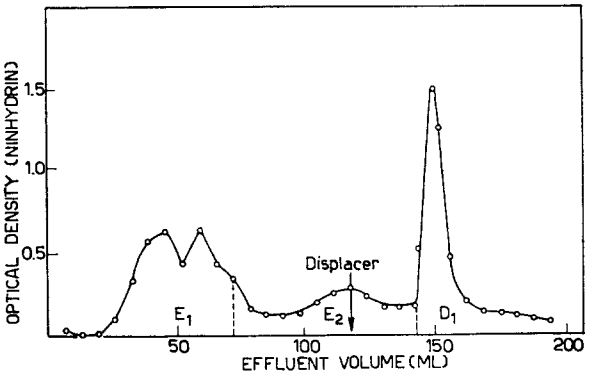

Fig. 5. Fractionation of a peptic digest of an ACTH preparation in a charcoal column. Eluant, a mixture of 0.5% octanol and 0.1% decanol; displacer, a saturated decanol solution. For experimental details, see text.

TABLE III

NITROGEN AND ACTIVITY DISTRIBUTION OF FRACTIONS FROM VARIOUS CHARCOAL COLUMNS*

Fractions	*Nitrogen*	*ACTH activity*
	%	USP units/mg
E_1	55	< 6
E_2	22	5–15
D_1	20	150–200
E_1'	55	300–350
D_1'	36	30–50
E''	95	
D''	4	

* See Figs. 5, 6, and 7.

20 mg of material from the displacement fraction (D_1) collected from several runs was dissolved in 35 ml of a mixture of 0.7% octanol and 0.1% decanol and pressed into a column (section combination: 14, 6.5, 5, 3.5, 3.5 ml) prepared with the adsorbent in equilibrium with the solution. The new elution and displacement peaks were surprisingly well-resolved (Fig. 6); the former fraction (E_1') contained the bulk of the activity, as anticipated (Table III). Several experiments using peptides from different batches but prepared by the same methods gave very close to the same results.

In order to confirm the effectiveness of the resolution into two fractions, the material in the elution fraction (E_1) was subjected to a new analysis under the same conditions as the first run; only a very small displacement peak (D'') was obtained (Fig. 7).

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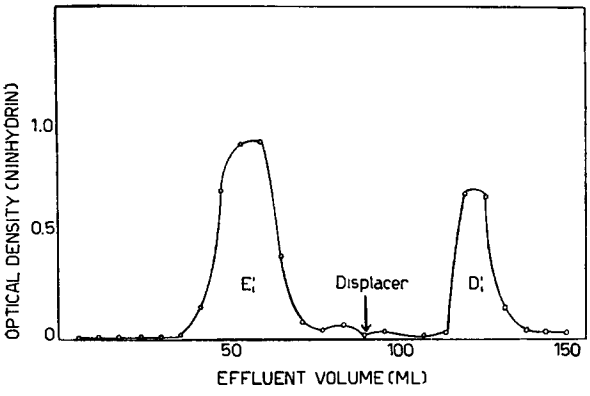
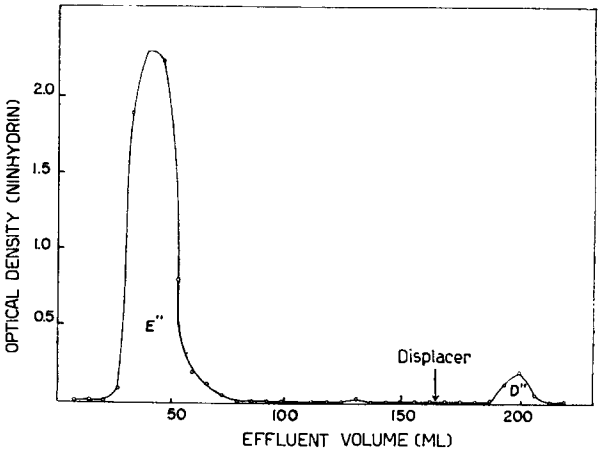


Fig. 6. Refractionation of a displacement fraction (D_1) in a charcoal column. Eluant, a mixture of 0.7 % octanol and 0.1 % decanol, displacer, a saturated decanol solution. For experimental details, see text.

Fig. 7. Rechromatography of an elution fraction (E_1) in a charcoal column. Same conditions as in Fig. 2.



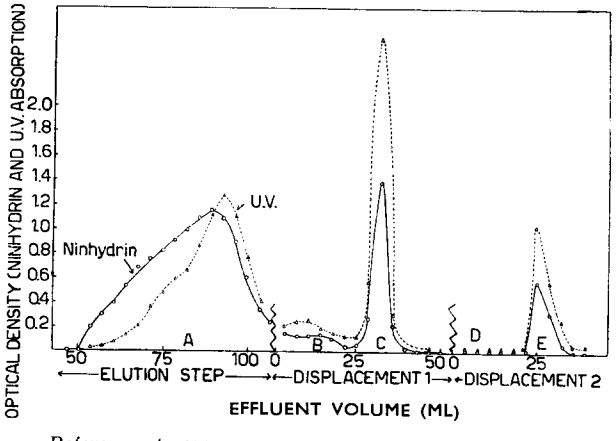


Fig. 8. Sectional displacement analysis of a peptic digest of an ACTH preparation in a charcoal column. Eluant, 0.4 % decanol solution; displacer, a saturated solution of decanol. For experimental details, see text.

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In a typical experiment with decanol as saturator, 60 mg of a peptide preparation was dissolved in 50 ml of 0.4% decanol and pressed into a thoroughly prewashed column (4 sections each, 18 ml in volume). Elution was performed with 80 ml of the 0.4% decanol solution. The column sections were then broken apart; the two lower and the two upper sections were displaced separately with saturated decanol solution (Fig. 8, displacement 1 and 2). Fig. 8 shows the distribution of the material; Table IV contains data presenting the average N-distribution and the assay values from all experiments done by this method. It is evident from Table IV that the C fraction, which was located in the first displacement peak, has the highest ACTH potency, amounting to 200–250 U.S.P. units per mg. This represents about a 5-fold purification of the starting material.

TABLE IV

NITROGEN AND ACTIVITY DISTRIBUTION OF FRACTIONS OBTAINED FROM CHARCOAL COLUMN USING SECTIONAL DISPLACEMENT ANALYSIS*

*Fraction**	*Nitrogen*	*Activity*
	%	USP units/mg
A	58	10
B	6	80–90
C	14	200–250
D	4	50
E	8	30

* See Fig. 8.

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SUMMARY

It has been shown that the properties of charcoal with respect to adsorption may be modified by pretreatment with a saturator in order to achieve satisfactory chromatographic resolution of the substances to be analysed. The bulk of the more strongly adsorbed material can be separated from other components by *sectional displacement* and/or rechromatography on more highly saturated columns. The concentration of the saturator must be fixed close to the *critical saturation point*, which is the concentration of the saturator above which the highly adsorbed component begins to appear in the eluate. By this procedure, elution and displacement analyses of insulin have been performed, whereby it was demonstrated that the hormone exhibits chromatographic homogeneity. Adrenocorticotropic peptides have also been investigated, and it has been found that a 5-fold purification from a starting material having a potency of approximately 50 U.S.P. units per mg can be achieved by the new procedures herein described.

RÉSUMÉ

Le pouvoir adsorbant d'un charbon peut être modifié par prétraitement avec une substance saturante de façon à permettre la séparation chromatographique satisfaisante des substances à analyser. La majeure partie des corps les plus fortement adsorbés peut être séparée des autres constituants par "*sectional displacement*" et/ou rechromatographie sur des colonnes contenant plus de

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substances saturantes. La concentration de la substance saturante doit être voisine du "*point critique de saturation*" qui est la concentration de la substance saturante au-dessus de laquelle le corps le plus fortement adsorbé commence à apparaître dans l'éluat. Par cette méthode, des analyses par élution et déplacement de l'insuline ont pu être effectuées, et ont montré que cette hormone est chromatographiquement homogène. Des peptides adrénocorticotropiques ont également été étudiés, et les auteurs ont réussi à obtenir un produit 5 fois plus actif que le matériel de départ, qui renfermait environ 50 unités U.S.P. par mg.

ZUSAMMENFASSUNG

Es wurde gezeigt, dass die Eigenschaften von Holzkohle hinsichtlich der Absorption durch eine Vorbehandlung mit einer saturierenden Substanz (Saturator) verändert werden können um eine befriedigende Trennung der zu analysierenden Substanzen zu erreichen. Die Hauptmenge des stärker absorbierten Materials kann von den übrigen Komponenten aus den einzelnen Segmenten der Säule dann isoliert verdrängt werden ("*sectional displacement*") und gegebenenfalls durch erneute Chromatographie auf mehr gesättigten Säulen weiter aufgetrennt werden. Die Konzentration des Saturators muss nahe dem *kritischen Sättigungspunkt* liegen, d.i. die Konzentration des Saturators oberhalb der die stark absorbierte Komponente gerade im Eluat aufzutreten beginnt. Mit diesem Verfahren wurden Elutions- und Verdrängungsanalysen des Insulins durchgeführt und dabei gezeigt, dass das Hormon unter diesen Bedingungen chromatographisch homogen ist. Adrenocorticotrope Peptide wurden ebenfalls untersucht und es wurde gefunden, dass die fünffache Reinigung eines Ausgangsmaterials mit einer Stärke von ungefähr 50 U.S.P. mit dieser neuen hier beschriebenen Methode erreicht werden kann.

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