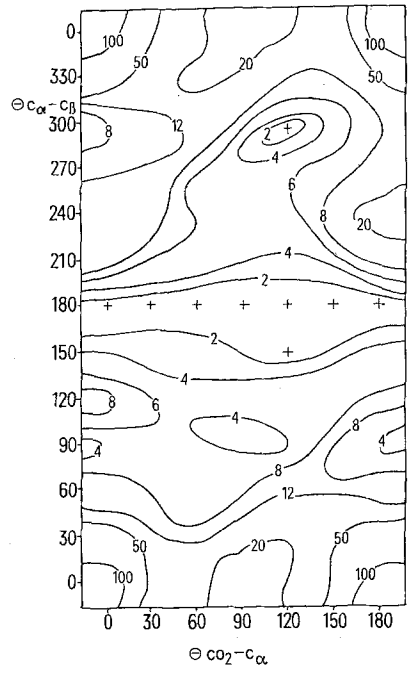

Fig. 1. Calculated energy vs. angle contour map for β-hydroxy GABA. The figure shows the relationships for the carboxylate rotation, θ co_2-c_α, and the α and β carbon relationship, θ c_α-c_β.

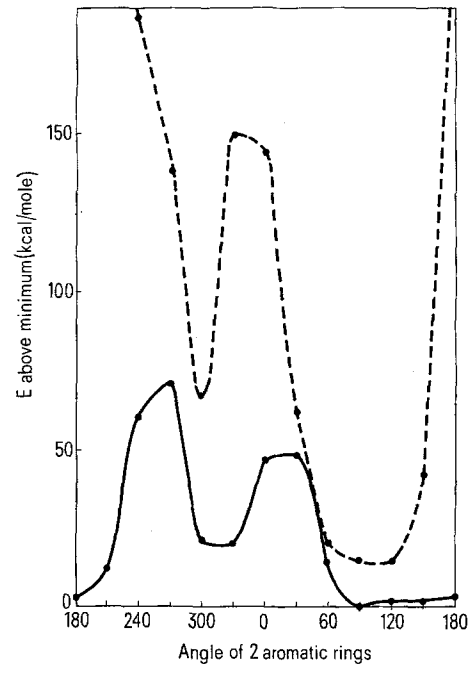

Fig. 2. Calculated energy vs. angle plot for the ring juncture bond in bicuculline. The dashed line is the energy for the proton-lactone *trans*-isomer. The solid line is the energy for the proton-lactone *cis*-isomer.

The results of the calculations on β-hydroxy GABA revealed two equivalent conformational preferences, Figure 1. The first conformation finds the molecule in an all trans conformation (θ c_α-c_β = 180°) and the carboxylate group free to rotate (θ co_2 — c_α = 0° — 180°). The second conformation is that in which the C_α–C_β bond is gauche (θ c_α — c_β = 300°) with the hydroxyl group close to the carboxylate group. The carboxylate group is inclined toward the hydroxyl group in a single conformation (θ Co_2 — C_α = 120°). This hydroxyl-carboxylate proximity way well be due to hydrogen bonding. This second preference resembles the folded conformation found for the GABA crystal[3]. In the trans conformation the onium to carboxylate oxygen distance is identical to that found for GABA, 5–6 Å depending upon the carboxylate oxygen considered[11].

The calculations on bicuculline were made on the protonated molecule. The configuration used was that derived by NMR analysis, Figure 2. There remains the ambiguity of the position of the proton on the nitrogen atom. It was necessary to perform calculations in which the proton was *cis* and *trans* to the lactone ring. The calculated preferred conformation occurs when the proton is *cis* to the lactone ring and with the dihedral angle between the aromatic rings 90°, as shown in (II) and Figure 2. In this conformation, the onium group to carbonyl oxygen atom is about 5.6 Å. If we assume that these features are the receptor equivalents of the onium group and carboxylate oxygen in GABA, then it is apparent, that bicuculline possesses structural features and dimensions which could impart receptor activity to it as a competitive antagonist.

The results of these calculations support our GABA pharmacophore hypothesis[2].

Zusammenfassung. Mit Hilfe der MO-Theorie wurden bevorzugte Konformationen für den GABA Agonisten β-Hydroxy-GABA und den Antagonisten Bicucullin berechnet. In beiden Fällen ergeben sich fast gleiche Abstände zwischen der Ammoniumgruppe und dem Sauerstoffatom wie in der entsprechenden Struktur von GABA selbst.

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PRO EXPERIMENTIS

Preparative Block Electrophoresis Using Sephadex G-25

Considering the widespread application of Sephadex for preparative gel-filtration, it is somehow surprising that so few attempts to use this material as support medium for preparative electrophoresis have been reported[1–6]. For this purpose, Sephadex G-25 appears to be the ideal grade, since it could be expected that most proteins would be excluded from the particles, separation being primarily determined by charge alone, and recovery of

proteins should be extremely simple. Nevertheless, of the 6 above-mentioned reports, only 2 describe methods based on the use of this Sephadex grade[1-6].

Recently described methods of preparative electrophoresis using Sephadex take place in columns, with the consequent problems in the elution of separated fractions[5,6]. The purpose of the present report is to describe a simple technique for preparative electrophoresis in blocks of Sephadex G-25 where elution of separated proteins is accomplished by cutting slices of gel and pressing out the protein-containing buffer in a syringe. Recoveries in this method range from 60 to 100% of the applied sample.

Material and methods. Sera and purified proteins. Two whole sera were studied, one from a normal individual, the other from a Waldenstrom's macroglobulinaemia patient.

Twice crystallized human albumin was obtained from Fluka. One IgG_3 monoclonal protein was isolated by the method previously described[7]. A digest of this protein was prepared using papain (Sigma). The enzyme was activated by incubation at 37 °C with 5 m*M* dithioerythri-

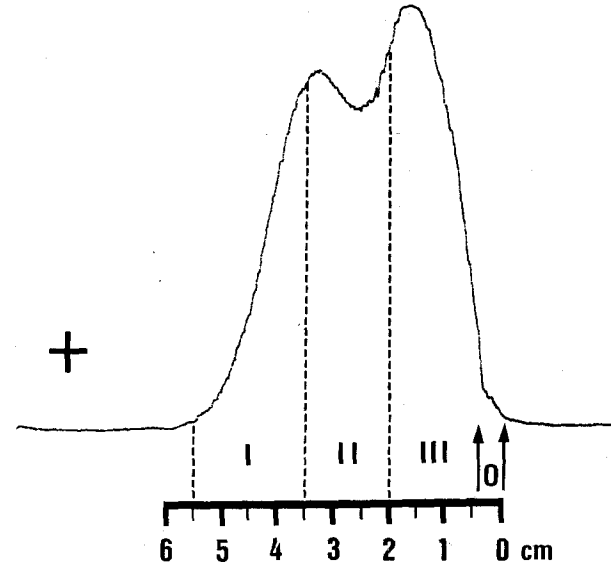

Fig. 2. Electrophoretic separation of a papain digest of a human IgG_3 protein. The separation profile was obtained by scanning the cellulose acetate imprint on a Joyce Chromoscan. The vertical arrows point to the limits of the sample trough (0). The distance migrated by the fractions is shown by the scale. 3 fractions were cut according to the dashed lines, and numbered from the anode.

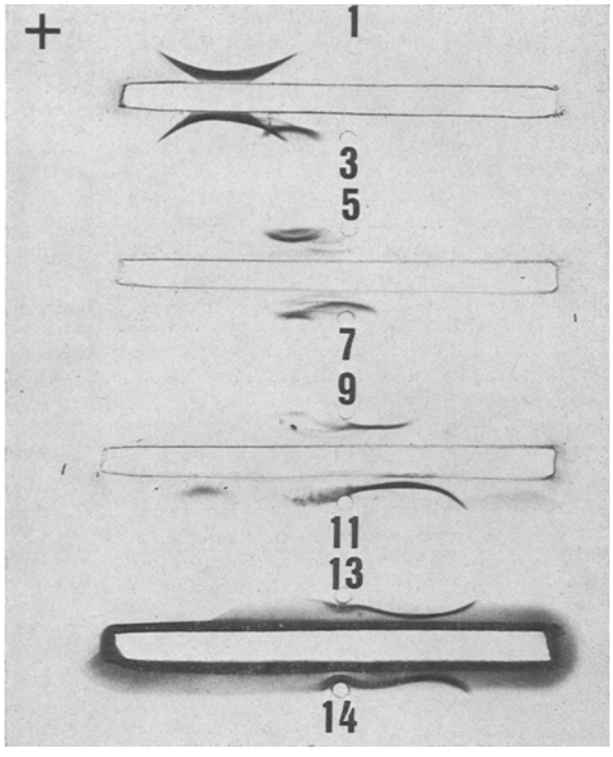

Fig. 1. Immunoelectrophoretic characterization of fractions separated by preparative electrophoresis from normal serum. The fractions correspond to 0.5 cm width gel slices, numbered from the anode, and anti-whole human serum was used to fill the troughs.

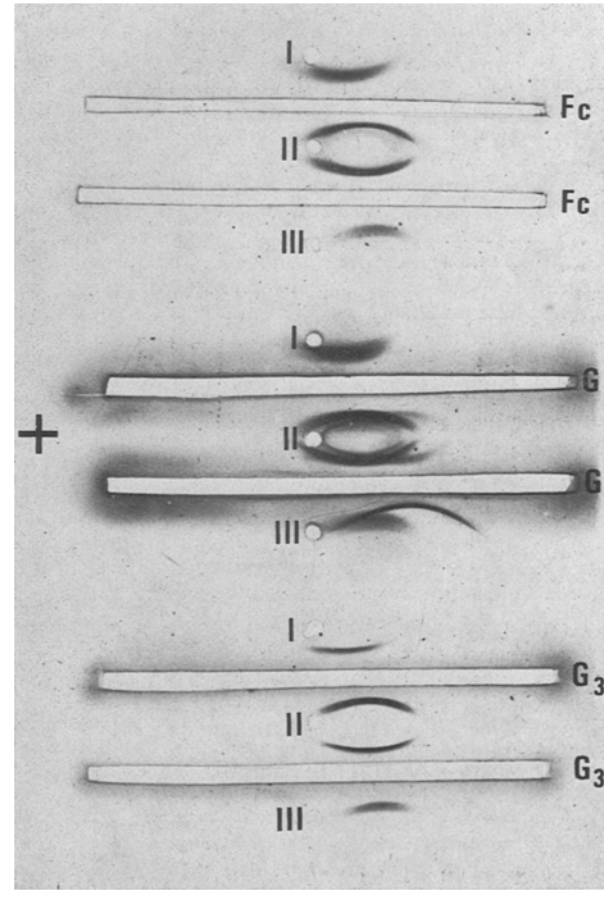

Fig. 3. Immunoelectrophoretic characterization of the fractions obtained by preparative electrophoresis of a papain digest of a human IgG_3 protein (see Figure 2). The following anti-sera were used: anti-whole IgG, able to react with Fab and Fc (G); anti-Fc from IgG (Fc), and anti-gamma$_3$ chains (G_3).

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Recovery studies on Sephadex G-25 preparative electrophoresis

Protein	Quantity at start (mg)		Quantity recovered (mg)		Recovery (%)		Volume recovered (ml)
	Biuret	O.D.	Biuret	O.D.	Biuret	O.D.	
HSA	10.6	11.2	11.2	11.0	—	98	2.5
HSA	10.8	11.8	8.2	11.7	81	99	1.9
H-IgG	3.5	4.2	2.1	2.8	60	65	2.1
H-IgG	7.0	9.0	3.0	5.0	42	55	1.7

Abbreviations: O.D., optical density (at 280 nm); HSA, human serum albumin; H-IgG, human IgG. Samples were applied as a mixture of 0.3 ml of protein solution and about twice that volume of buffer-equilibrated Sephadex G-25.

tol during 60 min, and added to the protein at a ratio of 1% (w/w). Digestion was allowed to proceed for 4 h, and stopped by adding 20 m*M* iodoacetamide.

Preparative electrophoresis. Sephadex G-25, medium grade, was equilibrated with barbital-boric acid buffer, pH 8.6[8], and after degassing, poured into starch-gel electrophoresis perspex plates, $18.5 \times 5.0 \times 0.6$ cm[9]. The circuit was closed through filter paper wicks (Whatmann 3MM) one of their ends placed underneath the G-25 layer and the other dipping into the tank buffer. The tanks were filled buffer identical to that used for equilibration of G-25. After pourring the gel slurry into the tray, the power supply was switched on at 20 mA during 5 to 10 min, in order to obtain a reasonable degree of dryness of the gel. After this pre-run period, a transversal trough was carved at 6 cm from the cathodic end of the gel. A mixture of 0.3 ml of the sample to be separated and G-25 was used to fill the trough. When albumin was present in the sample, some bromophenol blue was added to track its migration. Electrophoretic separation took place at room temperature, for 5 to 6 h, at 20 mA. After completion of the run, the wicks were removed and a damp cellulose acetate membrane carefully layered over the gel surface. The membrane was removed after 5 min, rinsed in 7.5% (w/v) tricholoroacetic acid, and stained with Ponceau S. Satisfactory imprints of the separation were obtained and used as guides to the areas to be cut and eluted. For recovery studies, the whole area of the gel calculated to contain the protein was cut and transferred to a 5 cm^3 disposable syringe with a 18 gauge needle, and the gel was submitted to manual pressure until no buffer was seen dripping out the needle. When isolation of the components of a mixture was desired, the gel was cut into a series of parallel slices, of variable width, and each slice treated as above. Protein contents of recovered samples were assessed by the biuret reaction and by O.D. readings at 280 nm, using electrophoresis buffer as blank. Starting samples were similarly assayed when recoveries had to be calculated. The qualitative composition of samples was determined immunoelectrophoretically, using poly- and monovalent anti-sera. The Sephadex G-25 used for the separation was re-cycled by repeated washings, first with saline, and later with barbitone-boric acid buffer, and re-used.

Results. The immunoelectrophoretic characterization of the fractions obtained from a preparative electrophoretic run of whole serum from a normal individual is shown in Figure 1. As expected, the only proteins isolated in an apparently pure form were albumin and IgG, both having isoelectric points quite distinct from the rest of serum proteins. Similar results were obtained with a whole serum from a patient with Wadenstrom's macroglobulinaemia.

The resolution of the method can be better evaluated by the study illustrated in Figures 2 and 3. A 4-hour digest of an IgG_3 protein, obtained at 0.1% (w/w) papain:protein, was electrophoresed, and the 2 protein peaks separated in the run collected as 3 fractions (Figure 2). Those fractions were later characterized immunoelectrophoretically, as shown in Figure 3. The isolation of pure Fc in this case deserves to be stressed, since the electrophoretic mobilities of the Fab- and Fc-fragments of this particular protein were quite close.

Recovery studies were carried out with purified albumin and the isolated IgG_3 protein. Results are given in the Table. Recoveries were far better with albumin than with the IgG protein. The reason for this is not entirely clear, but one possible explanation may lie in the fact that albumin could be located through its binding of bromophenol blue, so that the area where the protein had migrated was obvious without need to prepare any imprints in cellulose acetate membranes, as we had to do for IgG. In those conditions, it was easier to delimite the area of gel to recover in the case of albumin, and losses might have been reduced by this fact.

Comments. The present method appears quite attractive as an alternative to most other currently used block electrophoresis methods by its simplicity and good recoveries. One limitation is the small amount of protein that can be separated in a single run, ranging from 10 to 25 mg in our most succesful attemps. However, if this method is reserved to a last stage in the purification of a given protein, the limitation of quantity is not of paramount importance, since large quantities are not always available or necessary for further studies.

Resumen. Se describe un método de electroforesis preparativa en bloques de Sephadex G-25. Su resolución és satisfactória, permitiendo aislar la albumina y la IgG a partir del suero normal, y el fragmento Fc de una IgG_3 digerida con la papaina. Las recuperaciones varian entre 50 y 60% para la IgG, y entre 80 y 99% para la albumina.

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[10] The author wishes to acknowledge the skilful technical assistance provided by Miss M. J. GONÇALVES DA SILVA.