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Note

Separation of hypoglycin A from leucine and other amino acids on Sephadex G-10

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Hypoglycin A (β-methylenecyclopropylalanine), subsequently called hypoglycin, is a non-protein amino acid found in relatively large amounts in the seed and unripe aril of the fruit of *Blighia sapida*[1]. To date, isolation from *B. sapida* of hypoglycin (the free amino acid as opposed to hypoglycin B, the γ-glutamyl dipeptide) has produced a product contaminated to varying degrees with other mainly hydrophobic amino acids[1–4] which are very difficult to remove. Leucine and hypoglycin in particular exhibit very similar chromatographic properties[2–4]. Hypoglycin can be estimated chemically in the presence of leucine[4–7], but only by procedures that destroy the hypoglycin. There has hitherto been no method by which hypoglycin can be unequivocally separated from leucine and isoleucine on a preparative basis. In order to obtain hypoglycin for metabolic experimentation free from other amino acids most investigators now isolate hypoglycin B, which can be separated on anion-exchange resin from other neutral amino acids and which after hydrolysis provides hypoglycin free of leucine and isoleucine[8]. However, hypoglycin B is less abundant than the free amino acid.

This paper describes a procedure by which amino acid contaminants can be removed from hypoglycin preparations without loss of hypoglycin. The method is based on the capacity of compounds with planar ring systems and/or π-electrons to adsorb to Sephadex G-10 (ref. 9).

PROCEDURE AND RESULTS

Sephadex G-10 was equilibrated with water or 50% ethanol at room temperature and packed into a column (150 × 1 cm I.D.) with a Wright base plate fitting designed to minimise mixing as eluent left the column. Samples of hypoglycin or other amino acids were dissolved in 0.5 ml of water or 50% ethanol, as indicated in Fig. 1, and applied to the column which was then allowed to run with a flow-rate of *ca.* 6 ml/h. Fractions of 1.0 ml were collected and portions analysed for ninhydrin-positive material[10]. The amount of amino acid in each fraction was determined from a standard curve. The size of sample taken for analysis was varied according to the amount of material loaded on to the column. As only small portions of each sample were required for analysis the remainder, containing amino acid in water or 50% ethanol, could be evaporated to dryness in a rotary evaporator and/or freeze dried.
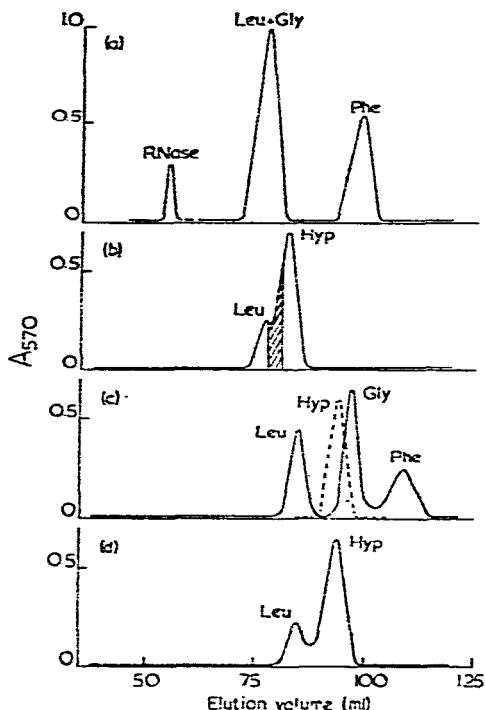
Fig. 1. Separation on Sephadex G-10 of various amino acids. In (a) and (b) the eluent was water, in (c) and (d) the eluent was ethanol–water (1:1). (a) A mixture of 10 μmol each of leucine, glycine and phenylalanine; (b) 50 μmol of impure hypoglycin, fractions collected as described in the text; (c) as in (a), the broken peak was a separate run of 50 μmol of hypoglycin prepared from hypoglycin B; (d) 50 μmol of impure hypoglycin. The hatched portion in (b) was the "leucine + hypoglycin" fraction described in the text.

The same column was used repeatedly for consecutive runs, though several pourings of the same G-10 were used throughout the work. The total volume of the column (V_T) was *ca.* 144 ml. The void volume (V_0) was determined by use of Dextran Blue or ribonuclease and was *ca.* 55 ml with water as eluent and slightly smaller at 51 ml with 50% ethanol. V_i (internal volume) + V_0 was determined with tritiated water and was *ca.* 105 ml with water and slightly larger at 113 ml with 50% ethanol.

Gas chromatography (GC) was kindly carried out by Professor G. W. Perold of the Department of Chemistry. Details of the procedure are in the legend of Fig. 2.

Samples of hypoglycin were kindly provided by Drs. D. Billington and H. S. A. Sherratt, Department of Pharmacology, University of Newcastle-on-Tyne, and Professor L. Fowden, Department of Botany, University College, London (Great Britain). Sample I had been extracted as hypoglycin A and was known to be impure. From the various analyses described below it was concluded to contain (%) hypoglycin 71, leucine 12, isoleucine 7, valine 2, phenylalanine 2, tyrosine 6. The other samples were derived from hypoglycin B and both on regular (Beckman 116) amino acid analysis, which does not resolve leucine and hypoglycin, and on gas chromatography showed a single peak.
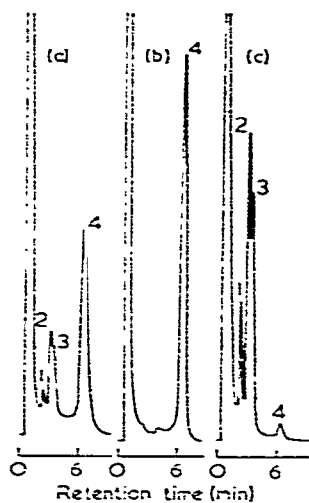
Fig. 2. Analysis by gas chromatography of a hypoglycin preparation before and after analysis on Sephadex G-10. Samples (*ca.* 1 mg) were dissolved in 17 μl of bis-N,O-trimethylsilylacetamide in 37 μl acetonitrile and were kept at 80° for 1 h. Samples of 1 μl were injected on to a 2.5 m × 2.6 mm I.D. column of 1% SE-30 on Anakrom, column temperature 120°, carrier gas nitrogen, inlet pressure 1 bar. (a) Hypoglycin sample prior to analysis on G-10; (b) the hypoglycin fraction prepared as described in text; (c) the "leucine" fraction separated from the hypoglycin. Approximate retention times (min) were: valine, 2.4; leucine, 3.1; isoleucine, 3.4; hypoglycin, 6.4 (peaks 1, 2, 3 and 4, respectively).

Fig. 1 shows the elution profile on Sephadex G-10 of several neutral amino acids when the eluent was either water or 50% ethanol. These solvents were convenient eluents because of their volatility. With water, leucine eluted with a distribution coefficient, K_D, of *ca.* 0.44 (Fig. 1a), earlier than and well separated from phenylalanine with a K_D of *ca.* 0.88. These results are similar to those of Eaker and Porath[9], who used 0.2 M acetic acid as eluent. Under our conditions glycine comigrated with leucine. Eaker and Porath[9] reported small differences in K_D between leucine, valine and glycine that were not detectable with our resolving power.

Fig. 1b shows the elution profile in water of hypoglycin A (sample 1), known to be contaminated to 20% or so with other amino acids. The first smaller peak corresponds to leucine, isoleucine and valine, the second larger peak is hypoglycin. Though the molecular weight of hypoglycin is 10 units greater than that of leucine it elutes later, and this is interpreted to be indicative of some degree of adsorption. K_D is 0.54 and the volume of separation between the two peaks is *ca.* 5 ml.

The overall peak was split into fractions as indicated –"leucine", "leucine + hypoglycin" and "hypoglycin", the "leucine + hypoglycin" re-run and the "leucine" and "hypoglycin" portions of the new profile combined with the first "leucine" and "hypoglycin" fractions which were then run again. The recovered fractions were analysed by GC (Fig. 2). Fig. 2a is the original material, which is resolved into four peaks, identifiable, by comparison with runs of the pure amino acids (not shown), from left to right as valine, a doublet peak of leucine and isoleucine, and the major

peak of hypoglycin. Thus on GC, leucine and hypoglycin separate clearly under conditions in which leucine and isoleucine separate poorly.

Fig. 2b shows the GC profile of the purified hypoglycin peak from the Sephadex column. Contamination by other amino acids is minimal. Fig. 2c is the profile of the "leucine" fraction, showing the presence of leucine, isoleucine and valine. These components and the purity of the purified hypoglycin with respect to amino acids other than leucine were confirmed on the regular amino acid analyser. From the combined results the original composition was estimated as indicated above. The phenylalanine and tyrosine in the original sample separated from the hypoglycin and branched-chain amino acids on the G-10 column and would have eluted after the hypoglycin. They were not found in either of the purified fractions.

Although the difference in K_D for leucine, isoleucine and valine on the one hand and hypoglycin on the other made it possible to achieve a purification of hypoglycin, we sought conditions that might increase the difference in K_D for hypoglycin with respect to the branched-chain amino acids. Eaker and Porath[9] found that the K_D for both leucine and phenylalanine increased on addition of high concentrations of sodium chloride. It is possible that salt decreases the hydration of the amino acids and facilitates adsorption or interaction of the amino acids with the gel, both leucine and phenylalanine possessing a K_D indicative of adsorption in 2 M NaCl. We were reluctant to use high concentrations of salt because of the difficulty of its removal in preparative runs, so we studied instead the effects of 50% ethanol.

Fig. 1c shows that in 50% ethanol both leucine (K_D 0.55) and phenylalanine (K_D 0.94) elute somewhat later than in water. Moreover, under these conditions glycine elutes at a K_D (0.74) considerably retarded with respect to leucine, though valine remains inseparable from leucine. Fig. 1d shows that the elution of hypoglycin is also more retarded in 50% ethanol than in water (K_D 0.69), and that the volume of separation between the branched chain amino acids and hypoglycin increases from *ca.* 5 to 9 ml.

DISCUSSION

Eaker and Porath[9] calculated the bed height equivalent to a theoretical plate (H) in their work to be *ca.* 0.06 cm for most amino acids, but increasing to *ca.* 0.10 for the aromatic amino acids. They packed their column with the finest fraction of an initial 2-kg batch of Sephadex G-10. We have used regular Sephadex G-10 as supplied. Our runs also were analysed as fractions rather than continuously which prejudices a true assessment of the shape of the peaks. However, values of H in numerous runs have ranged from 0.05 to 0.09 cm for the non-aromatic amino acids, values which compare favourably with those found by Eaker and Porath[9]. These authors concluded that two substances could be adequately separated for many purposes when $V_{e1} - V_{e2} = 0.5(\beta_1 + \beta_2)$ which for hypoglycin and leucine is about 6 ml (V_e = elution volume, β = width of elution curve in ml at the height equal to the maximum height divided by 2.71). Thus the separation achieved in water is at the limit of the useful range. To achieve 99% separation $V_{e1} - V_{e2}$ should equal $0.8(\beta_1 + \beta_2)$ which in our case is *ca.* 9.5 ml, and is just about achieved when 50% ethanol is used as eluent.

We are not in a position to explain in molecular terms why inclusion of ethanol in the eluent "spreads out" the elution profile, but presumably it involves factors such as those discussed by Eaker and Porath[9] for the results with increasing salt concentrations. Eaker and Porath[9] suggested that by choice of suitable conditions it should be possible to separate any pair of amino acids on G-10, and this proves to be true for the hitherto almost inseparable leucine and hypoglycin[2-4].

Presumably the superimposition of leucine and hypoglycin on the usual amino acid analyser profile is coincidental. Scott et al.[5] showed that it could be avoided by use of appropriate operating conditions. A very similar profile to that of Scott et al.[5] was observed by use of a lithium citrate elution system[11,12]. GC offers an easy analytical means of detecting hypoglycin in the presence of leucine or vice versa, with a much clearer separation of the two amino acids than obtainable with the Dowex systems. However, neither procedure is suitable for preparative purposes.

GC indicated that hypoglycin samples available to us prepared from hypoglycin B were free of detectable branched-chain amino acid contamination. They also eluted on G-10 as a single symmetrical peak. However, measurement of the potential inhibitory effect of the various hypoglycin samples on the incorporation of labelled leucine and phenylalanine into tRNA by liver cytosol showed a decreased potency of even hypoglycin B samples after passage through G-10. Because of the high specific activity of the labelled amino acids used in such experiments, minute traces of contaminants might easily change the specific activity of the labelled amino acids in the system. Passage through Sephadex G-10 may well be a useful precaution in experiments of this nature to minimise possible contamination in hypoglycin samples prepared from hypoglycin B, because hydrolysis of the dipeptide leads to a certain amount of breakdown[3].

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