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Countercurrent Distribution Studies on Anterior Pituitary Growth Hormone

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Since the isolation of a growth hormone preparation which behaved as a homogeneous protein by electrophoretic, diffusion and solubility criteria (Li, Evans & Simpson, 1945), a number of biological activities have been attributed to this protein (for a review of these activities see Young, 1953). The question of whether or not the growth-promoting as well as the other activities are indeed properties of the protein itself, or whether they might reside in smaller molecules, becomes increasingly pertinent in view of the recent extensive work on the adrenocorticotrophic hormone and on the active principles of the posterior lobe of the pituitary gland.

Thus 'protein' adrenocorticotrophic hormone fulfils the same physical criteria of purity as growth hormone, but its biological activity can be separated from the bulk of the protein material by a variety of techniques (see Cortis-Jones, Crooke, Henly, Morris & Morris, 1950; Astwood, Raben, Payne & Grady, 1951; Dixon, Moore, Stack-Dunne & Young, 1951; Hess, Harris, Carpenter & Li, 1951). The same situation is found in the case of the posterior-pituitary principles. van Dyke, Chow, Greep & Rothen (1942) isolated a protein which contained pressor, antidiuretic and oxytocic activities in the same ratios as those of standard posterior-pituitary powder. This protein was homogeneous by ultracentrifugal and solubility criteria but showed some inhomogeneity by electrophoresis. Many workers, however, have been able to separate an oxytocic principle from an antidiuretic-pressor principle (see Kamm, Aldrich, Grote, Rowe & Bugbee, 1928; Irving & du Vigneaud, 1938; Potts & Gallagher,

1944), and both principles have recently been shown to be peptides of simple composition (Livermore & du Vigneaud, 1949; Pierce & du Vigneaud, 1950; Turner, Pierce & du Vigneaud, 1951).

In the present investigation, preparations of growth hormone have been subjected to countercurrent distribution in order to determine whether they would behave as a single substance under these conditions and to discover whether or not the growth-promoting activity could be separated from the bulk of the protein by this means.

EXPERIMENTAL

Growth-hormone preparations. Most of the growth hormone used in this investigation was prepared in this laboratory from ox pituitaries by a modification (Reid, 1952) of the method of Wilhelmi, Fishman & Russell (1948). The material thus prepared does not always appear homogeneous during electrophoresis at pH 9.5. In addition, 25 mg. of a preparation were kindly supplied by Prof. C. H. Li, Berkeley, California, U.S.A., to Prof. F. G. Young. It was stated that the material behaved as homogeneous when studied by zone electrophoresis, on a chromatographic column and by end-group analysis.

Counter-current distribution. The apparatus was a 54-tube, all-glass apparatus (Quickfit and Quartz Ltd.), similar to that described by Craig & Post (1949). The volume of each phase was 35 ml. The solvent system was 2-butanol:aqueous *p*-toluenesulphonic acid, the acid concentration being approximately 0.005 M or 0.0025 M. The system was prepared by equilibrating equal volumes of the acid and 2-butanol. The 2-butanol was redistilled and was low in peroxides when tested as described by Newton & Abraham (1950). Theoretical curves were constructed as described by Craig & Craig (1950). For distribution, the material was first suspended in 1-2 ml. of water and then treated with 33 ml. of the aqueous *p*-toluenesulphonic acid saturated with 2-butanol (lower phase). All preparations studied went completely into solution under these conditions. After the

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first equilibration with 2-butanol (upper phase), a stable emulsion formed. This broke on centrifuging but left a thin layer of material at the interface, which was removed with a capillary pipette. In some experiments it was found necessary during the first ten transfers to break the emulsions in the first two or three tubes by centrifuging. For subsequent transfers about 20–30 min. usually sufficed for complete breakage of emulsions. Unless otherwise indicated, distributions were performed at 14–18°.

Estimation of contents of each tube after distribution. Although weight determination is the most general and the most reliable method of obtaining a complete counter-current distribution curve, such a method is impractical where it is necessary to use extremely small amounts of material. In the case of distribution of small amounts of proteins it would seem that a most sensitive and inclusive method of analysis is a photometric ninhydrin procedure applied after hydrolysis of the protein fraction. The following method, similar to that of Newton & Abraham (1950), proved satisfactory. The phases in a tube were made miscible by addition of 4 ml. of ethanol, and 2 ml. samples were removed, placed in thin weighing shells (Craig, Hausmann, Ahrens & Harfenist, 1951) and evaporated to dryness. The sample in each shell was dissolved in 0.5 ml. of 6N-HCl, which was then sucked into a small piece of soft-glass tubing (int. diam. 5–6 mm.), drawn out to a long fine tip at both ends. The tubes were sealed off and heated at 110–120° for 16 hr. The tips of the constrictions were then broken off and the contents blown into a shell and evaporated to dryness. Each sample was dissolved in 0.5 ml. of water, transferred by capillary pipette to a test tube, and subjected to the photometric ninhydrin procedure of Moore & Stein (1948). After development of the colour, samples were diluted with 10, 20 or 30 ml. of diluent before being read in a colorimeter (Evans Electroselenium Ltd., Harlow, Essex) against suitable blanks. When 50 mg. of protein were distributed through fifty-three transfers, the samples analysed contained 0–150 μ g. of protein. The method was not absolutely quantitative because, to save labour, no rinsing was done at any stage. Since, with care, reproducible results can be obtained, the errors must be essentially constant.

In the determination of partition coefficients an indication of the amount of material in each phase was obtained by measuring the ninhydrin colour given by unhydrolysed material. With a system containing NH_4OH , the absorption at 275 $\text{m}\mu$. was measured.

Recovery of material for biological assay. To recover material for determination of growth-promoting activity, the contents of desired tubes were combined, brought to pH 5–6 by addition of 0.1N-NaOH and concentrated to 30–40 ml. at 0–10° in a rotary evaporator (Craig, Gregory & Hausman, 1950). If, during the concentration, the solution became cloudy, more NaOH was added until the solution was as clear as possible. The final pH of the concentrate was about 9. The solutions were then dialysed for 48 hr. against distilled water and freeze-dried.

Determination of growth-promoting activity. The increase in weight of hypophysectomized rats was used as a measure of growth-promoting activity (Li *et al.* 1945; Wilhelmi *et al.* 1948). Female rats (Wistar albino strain) were employed in most cases and were maintained on M.R.C. diet 41 (Short & Parkes, 1949). Tests were begun 15–21 days after hypophysectomy, and the rats were often used for a second test

several weeks later. Growth hormone (dissolved in 0.9% NaCl and brought to about pH 9) was injected subcutaneously for 9 days, and the average weight of the rats on the 9th and 10th days was compared with their average weight during the 3 days before injection.

RESULTS

Choice of solvent system

The partition coefficients of a growth-hormone preparation *A* in several pairs of solvents in single tubes were investigated. With 2-butanol and either 0.1% (v/v) or 1% (v/v) acetic acid, 0.001N-HCl or 0.1% (v/v) NH_4OH , the material remained almost completely in the aqueous phase. With 0.005M and 0.05M *p*-toluenesulphonic acid as the aqueous phase, however, approximately 50–60% and 70%, respectively, of this preparation went into the butanol phase.

Partition of growth-hormone preparations between 2-butanol and approximately 0.005M p-toluenesulphonic acid

Fig. 1 shows the results of a 53-transfer distribution of 50 mg. of growth-hormone preparation *B*. A small amount of material at the interphase was removed from the first tube. It can be seen from Fig. 1 that there are at least two distinct components. At the completion of the experiment the tubes in which emulsions formed were numbers 1–10 and 46–50, the emulsions in tubes 1–10 being the more stable. With respect to the major component, the curve calculated for a theoretical distribution agrees well with that obtained experimentally. Preparation *A* gave a curve similar in all respects, except that a greater percentage of material was found in tubes 1–10.

After the above distributions had been completed, 22 mg. of preparation *C*, provided by Prof. C. H. Li, were distributed through twenty-four transfers. In this case a negligible amount of material at the interphase was present in the first tube. Less trouble with emulsions was encountered, although emulsions formed in two groups of tubes as before, but the emulsion in the lower-numbered tubes was the less stable. The analysis of the distribution is given in Fig. 2*a* and shows that the percentage of the component found in the low-numbered tubes is only about 3% of the total material represented by ninhydrin colour.

The yield of material recovered from tubes 18–24 was 9.4 mg., approximately 44% of theory. In the distribution of preparation *A*, 15 mg. (approximately 55% of theory) were recovered in tubes 43–52. To make certain that this somewhat low recovery was not due to a loss of material during dialysis, a concentrated dialysate from another distribution was analysed by the ninhydrin method.

Fig. 1. Distribution curve of 50 mg. of growth hormone (preparation *B*) after fifty-three transfers. Solvent system—2-butanol:approx. 0.005M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density equal to optical density multiplied by 100 with correction made for dilution of samples. x, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0.04.

Fig. 2a. Distribution curve of 22 mg. of growth hormone (preparation *C*) after twenty-four transfers. Solvent system—2-butanol:approx. 0.005M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. x, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0.04.

From 180 mg. of starting material less than 1 mg. was found in the dialysate of the main component. The low yield was probably due to the material passing over into the trap during freeze-drying.

*Partition between 2-butanol and
0.0022M *p*-toluenesulphonic acid*

Although in the system with 0.005M *p*-toluenesulphonic acid the main component distributes in

Fig. 2b. Distribution curve of 14 mg. of growth hormone recovered from main component of the distribution of preparation *B* (Fig. 1) after sixteen transfers. Solvent system—2-butanol:0.0022M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. x, Theoretical values for a partition coefficient of 4.8.

good agreement with theory for a single substance, a better estimation of homogeneity could be made if conditions could be so chosen that the partition coefficient was close to unity. A preliminary experiment indicated that with an acid concentration of about 0.0025M, a partition coefficient of about unity was obtained. A 14 mg. sample of the material recovered from the main peak of the distribution of preparation *B* in the first solvent system was re-

Fig. 3a. Distribution curve of 40 mg. of growth hormone (preparation *E*) after twenty-four transfers. Solvent system—2-butanol:0.0044 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0.04.

Fig. 3b. Distribution curve of 40 mg. of growth hormone (preparation *E*) after twenty-four transfers. Solvent system—2-butanol:0.0022 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values for a partition coefficient of 3.8; partition coefficient of minor component, <0.04.

Fig. 4. Electrophoretic diagram of preparation *E*. pH 9.5, glycine buffer, ionic strength 0.1. A, B, C and D, 150, 185, 300, 480 min., respectively. Top: ascending boundary; bottom: descending boundary.

distributed in a system with 0.0022 M acid (the exact molarity was determined by titration of the aqueous phase before its equilibration with 2-butanol). The distribution curve is given in Fig. 2b and showed the partition coefficient to be greater than had been expected and that the curve had spread on both sides of the theoretical curve.

To examine the possibility that the deviation from the theoretical might have been due to degradation during the recovery of the material from the first distribution, 40 mg. of another growth-hormone preparation (*E*) were distributed through twenty-four transfers in the same system (0.0022 M acid). As a control, a second sample was distributed at the same time in 0.0044 M acid in the other half of the apparatus. The curves are given in Figs. 3a and 3b, and show that deviation from the theoretical again occurred at the lower acid concentration.

It is possible that the ninhydrin colour in the first few tubes might have been due to traces of ammonia passing into the counter current apparatus with the upper phase. The ammonia might then have been extracted by the lower phase. Determination

of the ninhydrin colour of samples from these tubes before and after hydrolysis showed the expected increase in colour following hydrolysis. Also the formation of emulsions in these tubes would be unlikely if the nitrogen present were only in the form of traces of ammonia.

A sample of preparation *E* was subjected to electrophoresis (Perkin-Elmer micro-electrophoresis apparatus, Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.) at pH 9.5, which is close to pH values used in the study of homogeneity of growth-hormone preparations (Li *et al.* 1945; Wilhelmi *et al.* 1948). This was carried out to see if the two components found in this preparation by counter-current distribution could be clearly separated by the electrophoresis. The pattern obtained is given in Fig. 4 and shows that no clear separation occurred.

*Biological activity of fractions
obtained by distribution*

Preliminary experiments showed that a fair proportion of biological activity could be retained when growth hormone was distributed between

2-butanol and 0.005M *p*-toluenesulphonic acid if dialysis were used as a means of removing the acid. The partition coefficient (*k*) of the biologically active material was then investigated. A 20 mg. sample of preparation *B* was equilibrated in a test tube between 20 ml. of each phase. In order to make sure that the activity was not concentrated in the material at the interphase, and was thus being discarded at the beginning of the distribution, this material was left with the lower phase, approximately 90% of the upper phase being drawn off. If the activity were in the main component (*k* of approx. 10, as shown by the distribution curves), a total of about 20% of the activity should remain with the lower phase and interphase fraction. These experiments were carried out at 0–4°. The assay results are in Table 1, section 1, and show that, at the dose employed, the upper-phase material gave twice the increase in weight as did the material from the lower phase. Comparison with a dose/response curve, determined with the less-pure preparation (*A*), indicated the activity of the lower-phase fraction to be about 20% of the upper-phase fraction.

Table 1. *Growth-promoting activity of preparations after distribution*

Preparation	No. of animals	Daily dose (μg.)	Mean increase in wt. (g.)	Standard error of the mean	Fisher's <i>P</i> relative to saline control
Section 1*					
Preparation <i>B</i> , control treated with 0.005M <i>p</i> -toluenesulphonic acid	3	100	19	±2.3	—
Preparation <i>B</i> , after equilibration between the two phases of the system, material from both phases	5	100	17	±1.3	—
Preparation <i>B</i> , treated as above, upper phase	4	100	20	±1.1	<0.001†
Preparation <i>B</i> , treated as above, lower phase plus 10% upper phase	5	100	9	±0.9	—
Section 2					
Preparation <i>A</i> (material from tubes 43–52), 53-transfer distribution	6	100	14	±0.8	<0.001
Saline control for above	3	—	–2	±1.4	—
Preparation <i>C</i> , untreated	4	50	22	±3.4	<0.02
Preparation <i>C</i> (material from tubes 18–24), 24-transfer distribution	4	100	21	±0.7	<0.001
Saline control for above	2	—	2	±1.5	—
Preparation <i>D</i> , untreated	4	50	17	±0.8	<0.01
Preparation <i>D</i> * (material from tubes 7–10), 9-transfer distribution	4	50	14	±0.9	<0.01
Saline control for above	2	—	0	±2.5	—
Section 3					
Preparation <i>E</i> , untreated	8	50	15	±1.4	<0.001
Preparation <i>E</i> (material from tubes 15–23), 24-transfer distribution, 0.0022M acid	{ 9 6†	{ 50 100	{ 9 15	{ ±1.4 ±1.3	{ <0.01 —
Preparation <i>E</i> (material from tubes 20–24), 24-transfer distribution, 0.0044M acid	9	50	10	±1.5	<0.01
Saline control for above	6	—	2	±0.5	—

* Experiments carried out at 0–4°; all other experiments at room temperature, 17–18°.

† Fisher's *P* of upper phase value relative to lower phase value.

‡ Experiments carried out on rats used first as saline controls for this section.

It was then found that samples isolated from each of the main peaks in three experiments with different preparations possessed growth-promoting activity. The results are given in Table 1, section 2. In another experiment inactivation apparently occurred.

Results of bio-assay of growth hormone distributed between 2-butanol and 0.0025M *p*-toluenesulphonic acid are given in Table 1, section 3, and demonstrate that the material recovered from the main peak retained a high proportion of activity.

DISCUSSION

The data show clearly that a large proportion of the growth-promoting activity of the preparations is found in the major component. The other component, however, has not been tested for biological action and it is possible that it may possess some activity. Although some loss of growth-promoting activity appears to take place during the distribution or recovery for assay, the material is relatively stable. In some experiments it was exposed to the solvent system for up to a week at 14–18°.

The molecular weight of growth hormone in the systems used for distribution is unknown. Recent measurements in phosphate buffer, pH 2.3, by Li & Pederson (1953) indicate a molecular weight of about 45 000, the same as in neutral solution. The fact that counter-current distribution can be successfully applied to growth hormone makes it appear that the technique will be of value in studies of the purity and perhaps in the isolation of other proteins of comparable molecular weight.

With insulin, Harfenist & Craig (1952) have demonstrated the existence of one major component with, in some preparations, varying amounts of closely related components. The solvent system employed was 2-butanol: 1% (0.077M) dichloroacetic acid. With the system, 2:4:6-collidine:water, Hess, Carpenter & Li (1952) showed that 'protein' adrenocorticotrophic hormone (mol.wt. 20 000) did not distribute as a single substance, and that biological activity was concentrated in a fraction of the material.

It is interesting to note the extremely low concentration of *p*-toluenesulphonic acid (0.005M) in the system in which growth hormone has a partition coefficient of about 10. A similar system with *n*-butanol as the upper phase has been used for the purification of vasopressin (Turner *et al.* 1951), but the concentration of *p*-toluenesulphonic acid necessary to give a partition coefficient of unity was 0.1M. With corticotropin *B* concentrates Kuehl, Meisinger, Brink & Folkers (1953) have obtained a favourable partition coefficient with 0.03M trichloroacetic acid; the same range of concentration of other organic acids was used in the distribution

studies of Payne, Raben & Astwood (1950) on adrenocorticotrophic hormone. The reason for the relatively high solubility of growth hormone in the organic phase with such a low concentration of the acid is probably related to the presence of the large number of basic groups (approx. 58, Li & Evans, 1948) in growth hormone.

In the system with an acid concentration of 0.005M the curve of the main component agrees well with theory for a single substance. However, the use of 0.0022M acid in an attempt to get a more favourable partition coefficient for study of homogeneity led to non-theoretical behaviour, the curve showing a tendency to spread on both sides of the theoretical curve. Three possible reasons for non-theoretical behaviour are the presence of other components or transformation products, a change in partition coefficient with changing concentration of the solute, or the fact that equilibrium in the distribution of the solute between the phases might not be obtained before each transfer (Craig & Craig, 1950). It seems unlikely that the last two reasons would apply only at the lower concentration of acid. Further work in a different solvent system will be necessary to see if more than one component is represented by the main peak. It is possible that with an acid concentration of 0.0022M not enough acid is present to form the most stable possible complex of *p*-toluenesulphonic acid with the many basic groups of growth hormone. A number of complexes with slightly different partition coefficients might then exist in equilibrium. At a concentration of 0.0022M acid, it is of interest that the number of acid equivalents in one tube is about equal to the number of basic equivalents in 50 mg. of growth hormone.

SUMMARY

1. Countercurrent distribution of preparations of growth hormone in the system 0.005M *p*-toluenesulphonic acid:2-butanol produced at least one major and varying amounts of a minor component. The best preparation, supplied by Prof. Li and stated to be homogeneous by electrophoresis, chromatographic column and end-group analysis, contained only 3% of the minor component.

2. Growth-promoting activity was present in the major component, the distribution curve of which agreed well with the theory for a single substance.

3. With 0.005M *p*-toluenesulphonic acid, the major component had a partition coefficient of about 10. A system with 0.0022M *p*-toluenesulphonic acid was used in an attempt to obtain a more favourable partition coefficient for the study of homogeneity of the major component. In this case the distribution curve of the major component was wider than the theoretical curve. Possible

reasons for this deviation from the theoretical are discussed.

4. A comparison between the distribution curve of a preparation of growth hormone and its electrophoresis pattern is presented.

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Transfer Reactions Catalysed by some Sucrase Preparations

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In view of recent observations on the action of sucrase preparations from various sources (mainly yeasts and moulds, e.g. see Edelman, 1950; Bacon & Edelman, 1950; Blanchard & Albon, 1950; Fischer, Kohtès & Fellig, 1951; Bacon, 1952; Wallenfels & Bernt, 1952; White & Secor, 1952; Bealing & Bacon, 1953; Bealing, 1953) the existence of enzymes capable of catalysing solely the hydrolysis of sucrose to glucose and fructose is becoming increasingly doubtful. It appears that the fundamental action of sucraes may be to transfer fructose residues to suitable acceptors with consequent production of mono-, di- and oligo-saccharides. Some of the implications of fructose transfer have been discussed by Edelman & Bacon (1951) and Bealing & Bacon (1953).

This paper describes experiments with yeast- and mould-sucrase preparations, involving the use of radioactive sugars, which experiments further elucidate the fructose-transferring capacity of these enzymes. Some of the results described here are an extension and amplification of those communicated to the Biochemical Society on 24 October 1952 (Edelman & Bealing, 1953).

MATERIALS AND METHODS

Enzyme and yeast preparations

Dialysed mould sucrase. This was prepared from *Aspergillus oryzae* or *Penicillium spinulosum* using the Hughes' bacterial press by the method of Bealing & Bacon (1953). Alternatively, a 'Takadiastase' preparation, obtained from