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Separation of Oestrogens in Urinary Extracts by Partition Chromatography

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(Received 30 March 1954)

In a method developed for the quantitative determination of oestrogens in urine (Bauld, 1954a) partition chromatography was applied to the separation and purification of oestrone and oestradiol-17 β . Selection of a suitable solvent system was based on the following considerations. The desirability of a system with the oestrogens partitioning markedly in favour of the stationary phase is obvious from a rearrangement of the equation of Butt, Morris, Morris & Williams (1951) to express retention volume in terms of the other factors. In addition, for a routine method it is preferable to have the oestrogens freely soluble in one or other of the phases. Investigation of the contaminants of the oestrone-oestradiol-17 β fraction of male urinary extracts (Bauld, 1954a) showed the presence of acidic and basic material, and this made it desirable to have aqueous alkali as the stationary phase. Carol, Haenni & Banes (1950) reported the use of an aqueous sodium hydroxide-benzene system for the separation of the oestradiol epimers, and this system was adopted, since it met the requirements noted above, even though it was considered probable that a non-linear isotherm would result.

The ease of separation of oestriol from oestrone and oestradiol-17 β by partition between water and benzene (cf. Bachman & Pettit, 1941) rendered the use of chromatography unnecessary for this pur-

pose. Oestriol was removed from the benzene solution of phenols and neutrals with water (Bauld, 1954a) and separated from the hydrophilic contaminants in this fraction by a partition chromatogram with 70% methanol-ethylene dichloride, a solvent system discovered empirically.

The factors influencing the elution pattern of both chromatograms were investigated in detail.

METHODS

Purification of materials

Reagents used were A.R. unless otherwise stated. The final distillation of solvents was done in all-glass apparatus.

Benzene. This was refluxed for 30 min. with H₂SO₄ (50 ml./l.), washed with *n*-NaOH (B.P.) (4 $\times$ 0.1 vol.), water (to neutrality), dried over anhydrous Na₂SO₄, and distilled.

Ethylene dichloride. This reagent (I.C.I., metal or stone-ware containers) was washed with *n*-NaOH (B.P.) (3 $\times$ 0.1 vol.), water (to neutrality), dried over anhydrous Na₂SO₄, distilled, and redistilled within 24 hr. of use.

Methanol (I.C.I. refined). This was refluxed for 16-20 hr. with NaOH pellets (5%, w/v) and Zn dust (5%, w/v) and distilled twice.

Celite 535 (Johns Manville and Co. Ltd., London). This was partially calcined at 400° for 4 hr., stirred with an excess of HCl and allowed to stand overnight. The solid was washed with water until the washings were free of Cl⁻ (AgNO₃ test), Fe³⁺ (SCN⁻ test), and the pH was that of distilled water (glass electrode). The material was then dried at 110° for 48 hr., cooled in a vacuum desiccator and stored in containers with tightly fitting glass stoppers.

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Apparatus

The apparatus used for chromatography is illustrated in Fig. 1. Fractions were collected in tubes graduated at 2 ml.

Preparation of chromatograms

Equal volumes of the aqueous and organic solvents, at the temperature at which the chromatogram was to be run, were shaken together (1–2 min.) and allowed to stand until clear. The separated aqueous (stationary) phase was added in known volume to dry Celite in a beaker and stirred for 5 min. An excess of the organic (mobile) phase was then added to the damp Celite; a uniform slurry, obtained by thorough mixing, was added in small portions (enough to give 1–2 cm. of height of column) to an empty chromatogram tube supported with the open end below the surface of a few ml. of mobile phase in a test tube. The plunger (Fig. 1) was moved rapidly up and down the column below the surface of the solvent through the slurry until uniform particles were obtained. These were then packed in small segments (about 2 mm.) by a slow firm movement of the plunger which trapped some of the particles and compressed them into a pad. The most suitable size of hole in the plunger depended upon the interfacial tension of the slurry (cf. Martin, 1949) and holes of standard wire gauge 19 and 22 (1.02 and 0.71 mm.) were used for the NaOH–benzene and 70% methanol–ethylene dichloride systems respectively.

The process described above was repeated until the required height of column was obtained. Sufficient pressure

was exerted during packing to give a rate of flow of solvent (hereafter called the percolation rate) of 10–12 ml./hr. Before use, the percolation rate was measured by allowing 5 ml. of mobile phase to pass through the column under gravity. The top surface of the column was always covered with mobile phase except momentarily before the application of the solutes. For routine assay an excess of slurry was prepared and the chromatogram packed to a definite length.

Application of solutes and elution

The substances to be separated were dissolved in 1 ml. of mobile phase, transferred to the top surface of the chromatogram, allowed to enter the column completely, and the procedure was repeated twice with 1 ml. washings of the original container. Mobile phase was then added and collection of the eluate begun. The effluent appearing during the application of the solutes is not included in the volume of eluate shown in this report, but must be added when retention volumes are being determined.

Colorimetric estimation

Solvent was removed from the chromatogram eluates by heating in a boiling-water bath in a current of air and the oestrogen content of the dry residue determined by the method already described (Bauld, 1954*b*), the colour correction being applied except in the case of oestrogen-free residues as indicated in the Results section.

Preparation of urine residues

The 'oestriol' and 'oestrone–oestradiol-17 β ' fractions of hydrolysed urine were prepared as described (Bauld, 1954*a*).

Determination of column parameters and partition coefficients

For these determinations a slurry containing a definite weight of Celite was packed in the column and the length of the chromatogram measured. The cross-sectional area of the inert phase (A_I) was determined from the weight of dry Celite used, its density (2.34), and the length of the column (L) (cf. Martin & Synge, 1941). The cross-sectional area of mobile phase (A_L) was determined from L and the retention volume of a dye with zero partition coefficient for the stationary phase (cf. Butt *et al.* 1951). The cross-sectional area of the stationary phase (A_S) was calculated by the difference between the cross-sectional area of the empty chromatogram and the sum of A_I and A_L . The value should agree within the limits of experimental error with the theoretical value calculated from the volume of stationary phase added to the Celite; if significantly lower, stationary phase is being lost from the chromatogram. The partition coefficient was calculated from the equation of Butt *et al.* (1951).

RESULTS

Separation and purification of oestrone and oestradiol-17 β

Fig. 2 shows the elution pattern of oestrone and oestradiol-17 β from a chromatogram with N-NaOH as the stationary and benzene as the mobile phase. Also shown is the eluate from a similar

Fig. 1. A. Solvent reservoir with capillary outlet and B19 cone-joint. B. Chromatogram column (1 x 15 cm.) with B19 socket-joint. C. Plunger for packing chromatograms; the disk of the plunger should fit the column B closely.

chromatogram to which was applied the 'oestrone, oestradiol-17 β , neutral' fraction of male urine. In the second case a brown zone (1 cm. wide) was left on top of the column even after collection of 150 ml. of eluate. Excellent purification and separation were achieved, but the great delay in elution of

Fig. 2. Chromatography of chromogens of 'oestrone-oestradiol-17 β , neutral fraction' of male urine (on one column) and of pure oestrone and oestradiol-17 β (on a second column). The characteristics of both columns were: 1 $\times$ 13 cm.; mobile phase benzene, stationary phase 0.8N-NaOH; 0.9 ml. stationary phase/g. Celite; percolation rate 9–10 ml./hr. The concentration of male urine chromogens is expressed as oestrone and is calculated from uncorrected colorimetric readings.

Fig. 3. Chromatography of chromogens of 'oestrone, oestradiol-17 β , neutral fraction' of male urine (on one column), and of pure oestrone and oestradiol-17 β (on a second column). The characteristics of both columns were: 1 $\times$ 12 cm.; mobile phase benzene, stationary phase 0.8N-NaOH; 0.8 ml. stationary phase/g. Celite; percolation rate 10 ml./hr. The eluent was changed at $\uparrow$ to ethylene dichloride-benzene (3:1, by vol.). The concentration of male urine chromogens is expressed as oestrone and is calculated from uncorrected colorimetric readings.

oestradiol-17 β was undesirable for routine determinations. Accordingly, after collection of the eluate from 10–50 ml. (oestrone fraction) the eluent was changed to unequilibrated ethylene dichloride-benzene (3:1, by vol.). This procedure was used for a year; in all the chromatograms carried out during this time oestrone was eluted by 40 ml., and subsequently the solvent change was made at this point. Fig. 3 shows the results of chromatograms of oestrone and oestradiol-17 β , and the corresponding fraction of male urine, under these conditions.

Investigation of variables affecting the elution pattern

Normality of stationary phase. The elution of 150 μ g. of oestrone and oestradiol-17 β from chromatograms with different concentrations of aqueous NaOH is shown in Fig. 4. The retention volumes varied when the stationary phase was less than 0.7N. Increase in concentration to 1.0N gave the same elution pattern as was shown with 0.7N and 0.8N-NaOH. The stationary phase adopted was 0.8N-NaOH.

Temperature. Preliminary experiments indicated that the effects of temperature and of concentration of the stationary phase were interrelated. Fig. 5 shows the results of experiments in which both these factors were varied. With 0.1N-NaOH no separation was obtained at 21°, both oestrogens being eluted by 8 ml., whereas at 16° oestrone and oestradiol-17 β were just separable. With 0.8N-NaOH variation in temperature still had an effect on elution patterns but much less markedly. It

Fig. 4. Effect of alkali concentration of stationary phase on elution of oestrone and oestradiol-17 β . The characteristics of the four columns were: 1 $\times$ 15 cm.; mobile phase benzene, stationary phase aqueous NaOH of the concentrations shown, 0.9 ml. of stationary phase/g. of Celite; temp. 21 $\pm$ 0.5°; percolation rate 9–10 ml./hr. The eluent was changed at $\uparrow$ to ethylene dichloride-benzene (3:1, by vol.). x—x, 0.5N-NaOH; ○—○, 0.6N-NaOH; ●—●, 0.7N-NaOH; —, 0.8N-NaOH.

was apparent that with a 12 cm. column, the temperature should be kept below 20° to allow an adequate zero elution zone between oestrone and the fast-running chromogens. The partition coefficient of oestrone between 0.8N-NaOH and benzene decreases by 35% as the temperature is raised from 18 to 37°.

Fig. 5. Effects of alkali concentration of stationary phase, and of temperature, on elution of oestrone and oestradiol-17 β . The characteristics of the four columns were: 1 $\times$ 12 cm.; mobile phase benzene, stationary phase aqueous NaOH of the concentrations shown; 0.8 ml. of stationary phase/g. of Celite; temp. as shown; percolation rate 9–10 ml./hr. The eluent was changed at $\uparrow$ to ethylenedichloride-benzene (3:1, by vol.). $\times$ — $\times$, 0.1N-NaOH, 21°; $\bullet$ — $\bullet$, 0.1N-NaOH, 16°; $\times$ — $\times$, 0.8N-NaOH, 21°; $\circ$ — $\circ$, 0.8N-NaOH, 16°.

Fig. 6. Chromatography of oestrone (25 μ g.) and of oestrone (25 μ g.) added to 'oestrone, oestradiol-17 β , neutral fraction' of male urine. The same column was used in both cases, the pure oestrone being run first. The characteristics of the column were: mobile phase benzene, stationary phase 0.8N-NaOH; 0.8 ml. of stationary phase/g. of Celite; temp. 18 $\pm$ 0.5°; percolation rate 10 ml./hr. All concentrations were calculated from corr. colorimetric readings. The parameters were: A , 0.9; A_L , 0.36; A_T , 0.18; A_S , 0.36 cm.²; L , 14 cm. (from 6 g. Celite). The partition coefficients were: 4.0 (theoretical); 4.4 (calc. for pure oestrone); 3.2 (calc. for oestrone in presence of male urine chromogens).

Amount of stationary phase added. Investigation of the optimum amount of stationary phase to be added to Celite showed, as expected from theoretical considerations, that the elution of oestrone and oestradiol-17 β was delayed as the concentration of stationary phase was increased from 0.6 to 1.0 ml./g. Celite. However, at the higher levels a marked tendency to trailing resulted. The slurry obtained under these latter conditions was visibly different, being granular, whereas that obtained with lesser amounts of stationary phase was flocculent. Initially a concentration of 0.9 ml. of stationary phase/g. Celite was used, and granular slurries with band-spreading were obtained unless rigid precautions were taken to keep the Celite dry (cf. Fig. 4). Use of 0.8 ml./g. of Celite was found to give the most satisfactory results.

Presence of urinary residues. Fig. 6 demonstrates that the retention volume of oestrone is decreased by the presence of the chromogens in the male urine fraction of 'oestrone, oestradiol-17 β and neutrals'. The retention volume of oestradiol-17 β is similarly affected by chromogens. This important factor has been overcome in the final method by taking 10–30 ml. as the oestrone, 40–90 ml. as the oestradiol, fractions, and as a frequent control procedure collecting the eluate from 8 to 10 and 30 to 40 ml. to demonstrate the absence of oestrogen. In the column shown, A_S and A_L were found to be equal (0.36 cm.²), with the calculated partition coefficient agreeing with the theoretical for pure oestrone but lower in the presence of the urinary contaminants.

Other variables. Oestrogens were eluted somewhat faster from columns packed to give a percolation rate of 20 ml./hr., with gravity feed. The calculated cross-sectional areas of such chromatograms showed the decreased retention volumes to be explainable on the basis of increased A_L/A_S resulting from the less firm packing. The percolation rate of 10–12 ml./hr. was selected for ease of reproducibility.

Figs. 2–6 demonstrate the tendency to trailing boundaries seen in this system. The partition coefficient of oestrone measured at concentrations of 10 and 1 mg./ml. shows a decrease (in favour of the aqueous alkali) of 25% at the higher levels.

Purification of oestriol

The chromatogram for the separation of oestriol from urinary contaminants was standardized at the outset. Initially the temperature was maintained at 25 $\pm$ 0.5° in a thermostatically controlled air enclosure. A series of experiments was conducted at intervals over a period of a year and the uniformity of elution patterns from 1 $\times$ 10 cm. columns is demonstrated in the scatter diagram (Fig. 7). For convenience in the final method the chromatograms are run in a room kept at 18 $\pm$ 1°. Fig. 8 shows a

typical separation of oestriol from urinary contaminants, and also the elution pattern of pure oestriol under the same conditions. The close agreement between the calculated and theoretical values for partition coefficient is shown.

DISCUSSION

The elution of oestrone and oestradiol- 17β described in this report differs from that found with the same solvent system by Swyer and his co-workers (Swyer & Braunsberg, 1951; Braunsberg, Stern & Swyer, 1952), in that their retention volumes are smaller and all three oestrogens are separated on the same column. Since they do not specify the height of their column, calculation of the cross-sectional area of the phases is not possible.

Their method is quick, but the zero elution zone between oestrone and oestradiol- 17β is small and oestriol is applied to the chromatogram at a time when its characteristics are markedly changing. One would expect purification to be poor, and this was demonstrated by Bitman & Sykes (1953). The latter investigators displace oestriol by passing carbon dioxide up through the column, a process which must cause gross distortion of the chromatographic zones. Moreover, the method of transferring oestriol in benzene (Bitman & Sykes, 1953) is unlikely to be quantitative.

The variation of elution pattern with alkali concentration of the stationary phase was to be expected from the known variation of partition coefficient under these circumstances (cf. Bachman & Pettit, 1941; Mather, 1942). The marked variation with temperature suggests that the solubility of alkali in benzene plays an important part in determining the distribution of the oestrogens. In instances where it is more convenient to control the temperature at higher levels, the separation between the zones can be maintained by increasing the column length.

The increase in retention volume of the oestrogens as the ratio of stationary to mobile phase increases is in accord with theoretical considerations. The irregular pattern obtained when 1.0 ml. of stationary phase/g. of Celite was used may have been due to the resultant increase in particle size. This would increase the time necessary for equilibration, and as pointed out by Glueckauf (1949) cause a tendency to channelling down the walls of the column. The percolation rate adopted in this investigation was 12–13 cm./hr. Howard & Martin (1950) used 18–44 cm./hr., but stated that separations were better with flow rates down to 10% of this. In the columns used, percolation rates of 10 ml./hr. were readily obtained without pressure by firm packing of the chromatogram. The conformity of the retention volume with theoretical values suggests that under

Fig. 7. Scatter diagram showing elution of oestriol from successive chromatograms. The characteristics of the columns were: 1 × 10 cm.; mobile phase ethylene dichloride, stationary phase 70% (v/v) methanol; 1.0 ml. stationary phase/g. of Celite; temp. $25 \pm 0.1^\circ$; percolation rate 10–12 ml./hr. The volume from 10–32 ml. was taken as the oestriol fraction in the routine analyses during this period.

Fig. 8. Chromatography (A) of oestriol (25 µg.) and (B) of oestriol (25 µg.) added to 'oestriol fraction' of male urine. The same column was used in both cases, the pure oestriol being run first. The column characteristics were: mobile phase ethylene dichloride, stationary phase 70% (v/v) methanol; 1.0 ml. of stationary phase/g. of Celite; temp. $18 \pm 0.5^\circ$; percolation rate 10 ml./hr. The concn. of eluate from 0 to 14 ml. was calculated from uncorrected colorimetric readings. The parameters were: A_L , 0.41; A_I , 0.17; A_g , 0.32 cm.²; L , 10 cm. (from 4 g. Celite). The partition coefficients were: 6.3 (theoretical); 6.5 (calc. for pure oestriol and oestriol in the presence of male urine chromogens). The break in B (oestriol + chromogens) is due to the fact that the colorimetric determinations of the eluate residues from 0–14 ml. (which are zero in terms of the colour correction) are shown as the uncorrected values to indicate the amount of non-specific oestrogen; the values from 16 ml. onwards are corrected.

these conditions equilibration is being reached. Slight variations between different workers in the technique of packing are inevitable. The important considerations are: uniformity of the chromatogram, demonstrated by the absence of channelling in dye studies, and obtained by exerting uniform pressure over the whole surface of the chromatogram as the segments are built up; maintenance of the ratio A_L/A_S at approximately unity, demonstrated by dye studies, and obtained by exerting very firm pressure during packing; reproducibility in the packing procedure, demonstrated by uniformity in the retention volume of the dye, and obtained by consistency in the amount of pressure exerted during packing. Once these have been standardized in a preliminary group of chromatograms packed for dye studies, control in routine analysis is obtained by checking the percolation rate and relation between weight of Celite in slurry and column length.

In order to ensure quantitative transfer of the residues to the chromatogram, the flask is washed 3 times with relatively large volumes of mobile phase. Since most of the solutes dissolve in the first wash, this is allowed to enter the chromatogram separately in order to minimize frontal analysis in the bulk of the solutes. The observed variation of partition with concentration is the probable cause of the trailing boundaries seen in the alkaline chromatograms (cf. Levi, 1949).

No investigation was conducted into the most suitable column dimensions. In elution analysis the concentration of solutes on the columns must be kept low, and in view of the large amounts of urinary contaminants a relatively large column was used. Dr S. M. Partridge (personal communication) recommended maintaining a height/diameter ratio of approximately 10/1. Glueckauf (1949) pointed out that low ratios lead to irregular packing and tilting of boundaries, while high ratios cause irregular elution owing to wall channelling.

The decrease in retention volume of oestrone in the presence of urinary contaminants suggests that these exert the displacement effect to be expected with aqueous alkali as the stationary phase (cf. Martin, 1949). The elution of oestriol is unaffected by the presence of urinary contaminants indicating a pure elution analysis.

With the variety of applications of the countercurrent principle now available for the separation of oestrogens in urine extracts, it is desirable to discuss the relative merits of the different methods. Stimmel's (1946) use of alumina was attractive because of the simplicity of operation of adsorption chromatography. The technique has not been widely used owing to the inability of other workers to reproduce his results (cf. Swyer, 1952), an inability probably due to variations in adsorbent

(cf. Bates, 1952). Engel (1950) summarized the theoretical and practical limitations of the use of alumina in quantitative methods. Nevertheless, Dr J. B. Brown (to be published), using methylated oestrogens, has produced a satisfactory reproducible separation by adsorption chromatography. The method described by Engel, Slaunwhite, Carter & Nathanson (1950), which uses a countercurrent liquid-liquid extraction train operating discontinuously, is of high precision and dependability, but the complex apparatus and lengthy procedure may prevent its general application to routine analyses. Moreover, oestrogens in urinary extracts are still markedly contaminated (cf. Slaunwhite, Engel, Scott & Ham, 1953). Migeon (1953), by additional distributions, effected further purification but lengthened the procedure.

The method described in the present report is inconvenient in that two partition chromatograms are involved, but the additional labour involved appears to be justified by the wide separation between the impurities and the oestrogens. Prof. C. Morris & Dr J. Preedy (personal communication) have separated the three oestrogens on one chromatogram by gradient elution but the method has not yet been applied to urinary extracts.

SUMMARY

1. A method for the separation and purification of oestriol, oestrone and oestradiol-17 β in extracts of hydrolysed urine is described.

2. Partition chromatography is used for the purification of oestriol and for the separation and purification of oestrone and oestradiol-17 β .

3. The principal factors affecting the elution of oestrogens from the partition chromatograms are described in full.

The author wishes to thank Professor G. F. Marrian, F.R.S., for his supervision of the work; Professor C. J. O. R. Morris for assistance in preparation of the report; and Mr J. Gordon for skilled technical assistance.

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A New Method of Large-Scale Preparation of Hypertensin, With a Note on its Assay

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(Received 15 September 1954)

The isolation and identification of hypertensin (Braun-Menendez, Fasciolo, Leloir & Munoz, 1939) or angiotonin (Page & Helmer, 1940) has so far been unsuccessful, and this paper describes a new method of preparation designed to produce large quantities of hypertensin for purification.

Hypertensin is formed during the incubation of the enzyme renin, derived from the kidney, with hypertensinogen, a substrate in the globulin fraction of the plasma. Owing to the low yield of hypertensin (probably not more than 1 mg./l.) it must be made on a large scale. Previous workers have reduced the large volumes necessary by salting out the plasma globulins before activation (Braun-Menendez, Fasciolo, Leloir, Munoz & Taquini, 1946). Finally the reaction has been stopped by precipitating the proteins with heat (Edman, 1945) or ethanol (Page & Helmer, 1940), and the filtrate concentrated by vacuum distillation. The renin hypertensinogen reaction is complicated by the concurrent destruction of hypertensin by 'hypertensinase activity' present in both serum and kidney extracts. The serum activity has been destroyed previously by acidification (Braun-Menendez *et al.* 1946), and it is possible to prepare renin with low hypertensinase activity (Pickering & Prinzmetal, 1938).

When starting volumes of 80 l. of blood are used all these described methods are cumbersome, and it has been found that rapid concentration of hypertensin can be achieved by adsorption on to charcoal from the serum and subsequent elution by glacial acetic acid. The hypertensinase activity of the serum does not affect the yield.

MATERIALS AND METHODS

The various types of kieselguhr, Celite 545 and Standard Supercel were supplied by Johns Manville Co. Ltd., Artillery Row, London, S.W. 1.

The charcoal for adsorption was 'animal charcoal technical powder', British Drug Houses Ltd. It was suspended in glacial acetic acid for a few hr., filtered by suction, washed with distilled water and methanol, and dried in an oven at 105°.

For the assay, urethane (British Drug Houses Ltd.), pentapyrrolidinium tartrate in polyvidone solution ('Anso-lysen Retard' May and Baker Ltd.), (-)-noradrenaline (Bayer Ltd.) and heparin 5000 units/ml. (Evans) were used.

Renin preparation. This was modified from that described by Pickering & Prinzmetal (1938). Rabbit kidney from animals killed 12-24 hr. previously was minced and stirred at 2° with ethanol (2 ml./g. of kidney) for 12 hr. The pulp was gauze-filtered and dried on filter paper at room temperature. This coarse material was powdered and kept its activity indefinitely in the cold (2°). Renin was extracted from the kidney powder by stirring it with 0.9% NaCl (10 ml./g. powder) at 2° for 12 hr. The pulp was filtered through gauze with squeezing and the cloudy filtrate clarified by filtering through a bed of Celite 545 after the addition of Celite 545 to the solution. The renin was precipitated by the addition of 40 g. of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. of filtrate and the precipitate allowed to settle at 2° overnight. The precipitate was filtered through a Whatman no. 50 paper, and the filter paper suspended in distilled water to dissolve the renin. This solution was then ready for use. The optimum amount of renin/l. of serum was discovered by a pilot experiment. (Usually the renin obtained from 150 g. of kidney powder was sufficient to treat 40 l. of serum.) Hypertensinase activity was very low in this renin preparation.

Hypertensin preparation. Stirred ox blood was obtained from the slaughter house in 80 l. batches, and the serum was separated in a no. 5 Alfa Laval milk separator. Frothing was stopped by the addition of a few drops of tributyl citrate or phosphate.

The 40 l. of serum were stirred with an electrically driven stirrer, and powdered animal charcoal was added (7 g./l.). After stirring for 10 min. the charcoal was allowed to settle for 10 min., then the supernatant serum was pumped through the Alfa Laval centrifuge to remove all suspended charcoal. This charcoal was discarded.