THE PURIFICATION AND THE AMINO ACID CONTENT OF VASOPRESSIN PREPARATIONS*

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Since the discovery (1) of the pressor activity of extracts of the posterior lobe of the pituitary gland, many laboratories have investigated the fractionation of the extracts obtained from this gland. The physiological potency of various preparations, as well as the methods of fractionation employed, have already been reviewed in considerable detail (2–4). In the present paper we report a new method of purification of vasopressin[1] which has yielded preparations of high potency. A study of the amino acid composition of these preparations is also presented.

Earlier investigations have shown the presence of certain amino acids in vasopressin preparations of high potency. In 1933 it was reported that a preparation having a potency of 200 units of pressor activity per mg. contained a relatively large amount of tyrosine and of sulfur (5). Later Irving, Dyer, and du Vigneaud (6), using electrophoretically purified material, found that after preliminary purification the tyrosine and cystine content increased with the pressor potency. They obtained the values 9.9 per cent (tyrosine) and 11.2 per cent (cystine) in their most potent preparation (200 units per mg.). Stehle and Frazer (7) described preparations having a potency of 200 units per mg. which contained 9.5 per cent tyrosine, 7.7 per cent cystine, and 8.9 per cent arginine. They reported that phenylalanine was lacking in their preparations, and that glutamic acid, aspartic acid, and leucine were probably lacking. Later, Stehle and Trister (8) reported the presence of proline and isoleucine in their preparations and the absence of tryptophan, glycine, histidine, and hydroxyproline. More recently Potts and Gallagher (9) reported a prep-

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[1] The pressor principle of the posterior lobe of the pituitary gland has been variously called pitressin, β-hypophamine, postlobin-V, and vasopressin. The last of these is used in the United States Pharmacopoeia, XIV, 1950, and will be used in the present paper.

aration having a potency of 450 units per mg. which contained 11.9 per cent tyrosine, 19.0 per cent cystine, and 12.5 per cent arginine.

The starting material (40 to 70 units per mg.) for the present investigation was prepared from desiccated posterior pituitary glands by the procedures of Kamm *et al.* (10) which effect partial separation of the pressor and oxytocic activities.

Attempts to purify vasopressin further by means of chromatography on Amberlite IRC-50 (Rohm and Haas Company) did not lead to sufficiently satisfactory results. Some purification was also obtained by means of counter-current distribution with the solvent system water-*sec*-butyl alcohol in the steel apparatus of Craig (11). The distribution constant of the pressor activity in this system was found to be about 0.15.

A preparation of vasopressin was then submitted to counter-current distribution at 10–13° in the system *sec*-butyl alcohol and 0.05 per cent aqueous acetic acid, with an all-glass distribution apparatus (12, 13). The peak tubes contained material which had a potency of 200 units per mg. Hydrolysis of this material and analysis for amino acids by the technique of Moore and Stein (14) revealed maxima at the positions for the amino acids shown in Table I, Sample 1. The peaks at the phenylalanine, tyrosine, proline, glutamic acid-alanine, aspartic acid, glycine, arginine, and cystine positions represented considerably larger amounts of amino acids than the others.

A more favorable distribution constant for counter-current purification was found with the system *n*-butyl alcohol and aqueous *p*-toluenesulfonic acid. In this system, the distribution constant can be adjusted over a wide range of values by varying the acid concentration. A value of 0.10 M for the acid gave rise to a distribution constant of about unity.

A part of the material obtained in the *sec*-butyl alcohol-0.05 per cent acetic acid run, which possessed an activity of 95 units per mg., was then distributed in the all-glass apparatus in the *n*-butyl alcohol-*p*-toluenesulfonic acid system. After 100 transfers, aliquots of alternate fractions were analyzed by the quantitative ninhydrin method of Moore and Stein (15). This showed a peak which proved to contain the pressor activity in the neighborhood of Tube 55. The *p*-toluenesulfonic acid was removed from the contents of the peak tubes by passage through a column of Amberlite IR-4B. Lyophilization of the eluate yielded a residue having a pressor activity of 300 units per mg. The major amino acid components, as shown in Table I, Sample 5, were phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, arginine, and cystine, and, in fact, their molar ratios, each to each, were approximately 1:1. The molar ratio of the ammonia present in the hydrolysate to any amino acid was about 3:1. From Table I it can be seen that only traces of other amino acids were present. The molar ratios are given in Table II.

TABLE I

*Composition of Vasopressin Preparations As Determined by Chromatography on Starch Columns**

The results are in mg. of constituent per mg. of unhydrolyzed material.

Constituent	Sample 1†	Sample 2†	Sample 3†	Sample 4†	Sample 5†	Sample 6†
Phenylalanine	0.051	0.098	0.029	0.110	0.103	0.114
Tyrosine	0.057	0.107	0.028	0.118	0.103	0.126
Proline	0.075	0.074	0.055	0.113	0.078	0.096
Glutamic acid or alanine	0.125	0.121	0.107	0.165	0.099	0.124
Aspartic acid	0.051	0.077	0.036	0.095	0.084	0.106
Glycine	0.033	0.051	0.035	0.064	0.051	0.060
Ammonia	0.089	0.028	0.013	0.031	0.037	0.036
Arginine	0.059	0.108	0.027	0.118	0.108	0.129
Cystine	0.073	0.115	0.025	0.129	0.134	0.149
Leucine + isoleucine	0.011	0.025	0.031	0.025	0.005	0.007
Valine + methionine‡	0.010	0.029	0.019	0.022	0.003	0.006
Alanine§					0.00	0.00
Threonine	0.008	0.014	0.012	0.013	0.00	0.00
Serine	0.015	0.017	0.017	0.014	0.007	0.003
Lysine	0.008	0.035	0.013	0.012	0.00	0.00
Histidine	0.00	0.014	0.003	0.00	0.00	0.00

* Values are not corrected for ash or moisture contents.

† Sample 1 was prepared by counter-current distribution with *sec*-butyl alcohol and 0.05 per cent acetic acid (380 transfers); potency, 200 units per mg. Sample 2 was prepared previously by electrophoresis (6); potency, 250 units per mg. Sample 3 was commercial pitressin; potency, 40 units per mg. Sample 4 was prepared by counter-current distribution (eleven transfers); potency, 240 units per mg. Sample 5 was prepared from material obtained in the distribution which yielded Sample 1. This was distributed with *n*-butyl alcohol and *p*-toluenesulfonic acid (100 transfers); potency of Sample 5, 300 units per mg. Sample 6 was prepared with Sample 4 as starting material. This was distributed through 100 transfers with *n*-butyl alcohol and *p*-toluenesulfonic acid; potency after lyophilization, 400 units per mg.

‡ Approximate values because peak emerges close to that of tyrosine (14).

§ These values are determined with the solvent system which separates alanine and glutamic acid (14).

TABLE II

*Molar Ratios between Constituents of Purified Vasopressin Preparations**

Constituent	Sample 5	Sample 6
Phenylalanine	1.01	0.93
Tyrosine	0.92	0.94
Proline	1.10	1.13
Glutamic acid	1.09	1.14
Aspartic "	1.02	1.08
Glycine	1.09	1.09
Ammonia	3.52	2.97
Arginine	1.00	1.00
Cystine	0.90	0.84

* Arginine arbitrarily chosen as 1.00.

Samples of each of the eight amino acids mentioned above, together with ammonium chloride, were mixed in molar ratios approximating those found in the vasopressin sample and chromatographed on starch. A plot of the micromoles of amino acid against the fraction number gave a graph similar to that constructed from the chromatogram data of the vasopressin hydrolysate.

We have also analyzed a sample of commercial vasopressin, "pitressin," (approximately 40 units per mg.) and a sample of vasopressin obtained several years ago by means of electrophoretic purification (6), which, when originally prepared, had an activity of 250 units per mg. The results are shown in Table I, Samples 3 and 2. It is clear that the eight amino acids which have now been found in the vasopressin samples prepared by counter-current distribution in the *p*-toluenesulfonic acid system were present in the electrophoretic preparation in amounts greater than those of the other amino acids present.

On the basis of these preliminary experiments a procedure, which is described in the experimental section, was devised which made it possible to obtain larger amounts of highly potent material for further study. A product was obtained which had a potency of approximately 400 units per mg. When this product was analyzed for amino acids, the eight amino acids previously cited were found to be present in molar ratios of approximately 1:1, together with about 3 moles of ammonia for 1 mole of any of the amino acids. Once again only traces of other amino acids were found. Qualitative tests were made which showed that tryptophan and hydroxyproline were absent. After correction for ash and moisture, the recovery in terms of amino acid residues and ammonia was 91 per cent of the weight of unhydrolyzed sample; the recovery of nitrogen was 87 per cent of the value obtained by the micro-Kjeldahl method. These results indicate that vasopressin very likely contains these eight amino acids and ammonia. Whether the portion unaccounted for is due to the presence of impurities or to other components in vasopressin so far not detected remains for future investigation. It should also be pointed out that the data do not preclude the possibility of the existence in this high potency preparation of substances of the same amino acid composition and similar distribution coefficient.

It is of considerable interest that six of the eight amino acids found in hydrolysates of our purified vasopressin preparations are the same as those found in hydrolysates of highly purified oxytocin preparations (16). Tyrosine, proline, glutamic acid, aspartic acid, glycine, cystine, and ammonia were found in each hormone preparation; in addition to these, leucine and isoleucine were found in the oxytocin preparation, while phenylalanine and arginine were present in the vasopressin preparation.

EXPERIMENTAL

Preliminary Experiments on Distribution of Vasopressin between n-Butyl Alcohol and p-Toluenesulfonic Acid Solution—Solutions of 0.30, 0.20, 0.10, 0.05, and 0.025 M *p*-toluenesulfonic acid (commercial grade, recrystallized from water) were equilibrated with equal volumes of *n*-butyl alcohol. An aqueous solution containing 50 mg. of vasopressin (potency, 60 units per mg.) per ml. of solution was prepared. Distributions were performed by shaking 10.0 ml. of equilibrated upper phase with 9.5 ml. of equilibrated lower phase and 0.5 ml. of the vasopressin solution. A linear relation between the distribution constant of pressor activity and the molarity of the acid solution was found to obtain in the region of 0.05 to 0.30 M, and a value of K close to unity was found at 0.1 M.

Preliminary Purification—Posterior pituitary powder U. S. P. (Lilly[2]) was submitted to the fractionation procedure of Kamm *et al.* (10). In this procedure a series of six fractions (*a* to *f*) is obtained by solution of the pressor activity, after partial separation from the oxytocin activity, in acetic acid and by addition of acetone and ether. Fractions *e* and *f* have the greatest pressor activity (40 to 70 units per mg.), as well as considerable oxytocic activity. The material from Fractions *e* and *f* was used in these studies.

Bioassay—The materials were assayed for pressor activity by measuring their effect on the arterial pressure of the cat, as compared with the effect of the standard posterior pituitary powder. The cats were anesthetized with sodium phenobarbital.

Removal of Oxytocin—Much of the oxytocin present in Fractions *e* and *f* was removed by taking advantage of the solubility of oxytocin in *sec*-butyl alcohol. A solution of the material (1 gm. in 25 ml. of 0.05 per cent aqueous acetic acid) was extracted six times with equal volumes of *sec*-butyl alcohol. Some vasopressin was also extracted from the aqueous solution by this treatment.

Counter-Current Distribution—The aqueous solution remaining after removal of the oxytocin was lyophilized. The residue was then submitted to counter-current distribution in a cold room at 10–13° with the use of separatory funnels. 9.8 gm. of the vasopressin preparation (45 units per mg., 440,000 total units) were dissolved in 100 ml. of equilibrated 0.09 M *p*-toluenesulfonic acid and introduced, together with 100 ml. of equilibrated *n*-butyl alcohol, into Funnel 1. After eleven transfers, the upper phases from Funnels 5, 6, and 7 were combined and extracted twice with 150 ml. of water to remove the vasopressin. The two aqueous extracts were added to the lower phases from the same funnels.

[2] The authors are indebted to Eli Lilly and Company, who generously made us a gift of some of the posterior pituitary powder used in this investigation.

Removal of p-Toluenesulfonic Acid—The aqueous solution obtained from the counter-current distribution, about 600 ml. in volume, was freed of acid by allowing the solution to pass through a column of ion exchange resin. A column 6 cm. in diameter and 20 cm. high of Amberlite IR-4B was used for 300 ml. of acid solution. The column was washed with 150 ml. of water.

The counter-current distribution and removal of acid were performed in the cold room during about 12 hours.

Intermediate Vasopressin Preparation—The solution of vasopressin was concentrated *in vacuo* at 0–10° to a small volume and finally lyophilized. The residue weighed 1.16 gm. and contained 240 units per mg., or 280,000 total units (63 per cent recovery). The results of the analysis for amino acids, according to the method of Moore and Stein (14), are given in Table I, Sample 4.

Preparation of Highly Active Material—A sample of 380 mg. of the intermediate vasopressin preparation was submitted to counter-current distribution in the all-glass machine (12, 13) in the cold room. Each tube contained 10 ml. of each phase of the *n*-butyl alcohol-*p*-toluenesulfonic acid system. After 100 transfers, the solutions from Tubes 51 to 60 were combined, separated, and treated in the manner described above to free them of acid. Assay of the solution showed that approximately 60,000 units of pressor activity were present. After lyophilization of the eluate, a residue of 120 mg. of amorphous, colorless powder remained. This would indicate an activity of 500 units per mg. for the material in solution before concentration and lyophilization. When the lyophilized residue was redissolved and assayed, a potency of approximately 400 units per mg. was obtained, which represents a total activity of 48,000 units (53 per cent recovery). The preparation contained 49.5 per cent carbon, 6.12 per cent hydrogen, and 19.5 per cent nitrogen (moisture- and ash-free). The ash content was 1.32 per cent.

Tubes 46 to 50 and Tubes 61 to 65 yielded 66 additional mg. of vasopressin preparation, with a potency of 320 units per mg. (21,000 total units, 23 per cent recovery).

Determination of Constituent Amino Acids—A sample of the preparation obtained from Tubes 51 to 60 was hydrolyzed and the hydrolysate was analyzed for amino acids. A plot of the micromoles of amino acid against the fraction number, constructed from data obtained with the starch chromatogram, is reproduced in Fig. 1. As this chromatogram showed no distinction between glutamic acid and alanine, another sample of the hydrolysate was chromatographed with the use of a solvent system in which a mixture of these amino acids is resolved (14). This second chromatogram showed that glutamic acid was present and that alanine was

absent. The results of analyses for amino acids are given in Table I, Sample 6, and the molar ratios to each other of the constituents are given in Table II.

The possibility of the presence of hydroxyproline and tryptophan was investigated. In a Hopkins-Cole reaction 5.0 mg. of the vasopressin preparation (potency 400 to 500 units per mg.) gave a negative test, while in a parallel experiment with 0.50 mg. of tryptophan the test was strongly positive. Furthermore, the per cent of absorption at 275 mμ in the ultraviolet absorption spectrum of the vasopressin preparation was quantitatively equal to the expected absorption due to the known tyrosine content. (Tryptophan and tyrosine both exhibit a maximum at 275 mμ.)

Hydroxyproline was not detected in several two-dimensional paper chromatograms, in which the *n*-butyl alcohol-acetic acid solvent of Block (17)

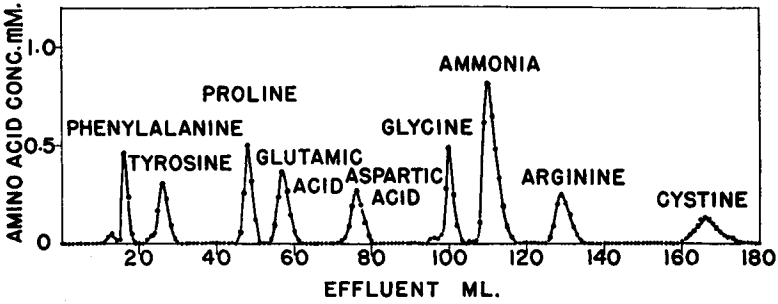

FIG. 1. Separation of amino acids from a hydrolysate of vasopressin. All conditions as previously described (16). Sample of hydrolysate placed on the column equivalent to 1.75 mg. of the unhydrolyzed vasopressin preparation.

and aqueous phenol (18) were used. The spots were developed with the isatin reagent described by Acher, Fromageot, and Jutisz (19).

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SUMMARY

A method for obtaining highly potent vasopressin preparations is described. This method consists of counter-current distribution in an all-glass apparatus with a solvent system of *n*-butyl alcohol and aqueous

p-toluenesulfonic acid and removal of the acid from the active fractions with an ion exchange resin. Preparations of high potency, 400 to 500 pressor units per mg., can be obtained by these procedures. Analysis of hydrolysates of such preparations by chromatography on a starch column shows the presence of phenylalanine, tyrosine, proline, glutamic acid, aspartic acid, glycine, arginine, and cystine in approximately equimolar ratios to each other. Ammonia was present in the hydrolysates in a molar ratio of about 3:1 to any one amino acid. Only traces of other amino acids were found. In terms of the 8 amino acid residues and ammonia, 91 per cent of the weight of the unhydrolyzed preparation was accounted for. Analyses of pressor material prepared by other means are also presented.

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