KONINKL. NEDERL. AKADEMIE VAN WETENSCHAPPEN — AMSTERDAM
Reprinted from Proceedings, Series B, 67, No. 1, 1964

ORGANIC CHEMISTRY

AMINO ACIDS AND PEPTIDES BOUND TO "VAN DYKE'S PROTEIN" IN THE PIG NEUROHYPOPHYSIS

BY

A. WITTER [1]), J. F. G. VLIEGENTHART and J. F. ARENS
Laboratory of Organic Chemistry of the University, Utrecht

(Communicated at the meeting of November 30, 1963)

SUMMARY

From an extract of pig neurohypophyses the "van Dyke's protein" can be obtained by gel filtration over Sephadex G25.

Lysine-vasopressin can be dissociated practically completely from this protein by treatment with 1 N acetic acid; oxytocin, however, is only partially dissociated. Under modified conditions it is possible to also dissociate the oxytocin practically completely.

By means of ion-exchange chromatography over Dowex 1X2 nearly pure lysine-vasopressin (overall yield 40 %) and somewhat less pure oxytocin can be obtained.

In addition to the peptide hormones many amino acids and peptides are being dissociated from the "van Dyke's protein" by 1 N acetic acid. These were separated by ion-exchange chromatography over Dowex 1X2 and paper chromatography and their amino acid compositions were determined.

INTRODUCTION

In isolating the neurohypophyseal hormones oxytocin and vasopressin, use has been made of an extraction and fractionation according to KAMM *et al.* [26] or an extraction of a protein-peptide hormone complex ("van Dyke's protein") [17], followed by a dissociation of this complex into a protein (neurophysin) and the peptide hormones.

The KAMM extract has been purified by counter-current distribution [34, 39, 40, 57, 58] and ion-exchange chromatography over Amberlite IRC 50 [30, 48, 56] or carboxymethyl cellulose [51, 59]. Other peptides present in the neurohypophysis can give rise to complications during this purification procedure.

A neurohypophyseal extract, obtained according to VAN DYKE *et al.* [17] under mild conditions, contains an apparently homogeneous protein, the so-called "van Dyke's protein", which possesses nearly all the oxytocic

[1]) Present address: Department of Pharmacology, University of Utrecht, Vondellaan 6, Utrecht.

and pressor activities of the starting material. The "van Dyke's protein" has been purified by precipitation with NaCl [3] or by gel filtration over Sephadex [41], a cross-linked dextran with molecular-sieve properties. The dissociation of the "van Dyke's protein" into the peptide hormones and the carrier protein neurophysin has been effected by means of trichloroacetic acid [4, 5, 6, 8, 15, 21, 31, 32], resulting in precipitation of the neurophysin, or with 1 N formic acid, followed by separation of the neurophysin from the peptide hormones by gel filtration over Sephadex [33, 42, 43].

Isolation of the neurohypophyseal hormones of different species led to the discovery of vasotocin [7, 9, 10, 22] and isotocin (= ichthyocin) [11, 23, 24, 50]. The existence of an unidentified oxytocic principle in amphibia has been demonstrated [19, 23]. Apparently some classes have characteristic peptide hormones. It is interesting that in some suiformes [18] both lysine- and arginine-vasopressin occur and that both WARD *et al.* [60] and SMITH and ROSENFELD [54] have evidence for the existence of another oxytocic principle apart from oxytocin in the monkey and the ox. The improvement of simple and mild techniques for the isolation of neurohypophyseal hormones seems useful.

We isolated "van Dyke's protein" from neurohypophyseal extracts by means of gel filtration on Sephadex. After the dissociation of the peptide hormones from the "van Dyke's protein", Sephadex was also used for the separation of neurophysin and these hormones. Together with the hormones other peptides and amino acids were liberated. This mixture could be separated by chromatography on the anion exchanger Dowex 1X2, applying volatile buffers [27, 46, 52]. Practically pure lysine-vasopressin and somewhat less pure oxytocin were easily obtained. This procedure is attractive for its simplicity and mildness and applicable if only small amounts of hypophyses are available. The rather drastic conditions normally used for the dissociation of the "van Dyke's protein" [4, 33], which may easily lead to artefacts, are obviously quite unnecessary (see also [3, 21]).

Some attention was paid to the components which, together with oxytocin and lysine-vasopressin, were associated with neurophysin in the original "van Dyke's protein" and set free by *mild* dissociation.

MATERIALS AND METHODS

Our thanks are due to Dr. S. Szpilfogel and Dr. J. D. H. Homan of N.V. Organon (Oss, Holland) for providing us with acetone-desiccated pig neurohypophysis powder.

Sephadex G25 (water regain 2.4) was ordered from Pharmacia, Uppsala, Sweden, and Dowex 1X2, 200–400 mesh, $Cl^{\ominus}$ form (Dow Chemical Co.) from Serva, Heidelberg, W.-Germany.

To all solutions used 1/10.000 volume of toluene was added as a preservative.

biological assay

The pressor activity in the rat was determined with the method of DEKANSKI [16]. Eighteen hours before testing vasopressin, the rats were injected intravenously with phenoxybenzamine.HCl, 1 mg/100 g body weight. As a working standard "pitressin" from Parke Davis Co. was used.

The oxytocic activity on the isolated rat uterus was determined according to HOLTON [25]. The uterus was suspended in a modified Locke Ringer solution without Mg". As a working standard, "piton" from N.V. Organon was used.

extraction of the acetone-desiccated pig posterior lobe powder

The extraction was carried out at 4° C with a 0.2 M pyridine–0.05 M acetic acid buffer pH = 5.9 [33]. A suspension of 95.3 g acetone-desiccated pig posterior lobe powder in 1 L pH = 5.9 buffer was stirred magnetically during 8 hours. After standing during another 16 hours without stirring, the suspension was centrifuged and the residue re-extracted thrice in the same way, with 500 ml pH = 5.9 buffer each time. Thus a residue B remained, which was lyophilized. The collected extracts were diluted with pH = 5.9 buffer to 4800 ml. The concentration of solid matter was about 1 % in this yellow-brown, opalescent solution A.

fractionation of the extract A over Sephadex G25

The solution A was filtered in 8 portions of 600 ml over a Sephadex G25 column (85 × 8 cm, 1000 g), which was equilibrated with the above mentioned pH = 5.9 buffer. The elution was carried out at room temperature with the same buffer at 400 ml/hr; fractions of about 13 ml were collected. From each third fraction 50 μl was pipetted and mixed with 2.0 ml of ninhydrin reagent [35]. After development of the colour and dilution with 8.0 ml 96 % ethanol : water = 1 : 1, the absorbancy at 570 mμ was measured (fig. 1). The high-molecular fraction A 1 and the low-molecular fraction A 2 were collected separately and lyophilized.

dissociation of the high-molecular fraction A1 with 1 M acetic acid and fractionation over Sephadex G25

Portions of 8 gram each of A1, were dissolved in 600 ml 1 M acetic acid and kept at room temperature during one hour. Then the solution was filtered over a Sephadex G25 column (82 × 8 cm, 1000 gram) which was equilibrated with 1 M acetic acid. The elution was carried out at room temperature with 1 M acetic acid at 500 ml/hr; fractions of about 16.5 ml were collected. From each third fraction 200 μl was pipetted and mixed with 2.0 ml of ninhydrin reagent [35]. After development of the colour and dilution with 8.0 ml 96 % ethanol : water = 1 : 1, the absorbancy at 570 mμ was measured (fig. 2). The high-molecular fraction A 1 a respectively the low-molecular fraction A 1 b were collected separately and lyophilized.

dissociation of the high-molecular fractions A 1 a and A 1 b

a) 500 mg of the fraction A 1 a were treated with 1 M formic acid and then filtered over a Sephadex G25 column (47 × 2.5 cm) as described by PORATH *et al.* [33]. The elution was carried out with 1 M formic acid at room temperature at 100 ml/hr; fractions of about 3.3 ml were collected. This gel filtration yielded a high-molecular fraction A 1 a 1 and a low-molecular fraction A 1 a 2, which were separately lyophilized and assayed for oxytocic activity.

b) 200 mg of the fraction A 1 a were treated with 1 M acetic acid and then filtered over a Sephadex G25 column (47 × 2.5 cm) as described for A 1. The elution was carried out at room temperature with 1 M acetic acid at 90 ml/hr; fractions of about 3 ml were collected. This gel filtration yielded a high-molecular fraction A 1 a 1′ and a low-molecular fraction A 1 a 2′, which were separately lyophilized and assayed for oxytocic activity.

c) 25 mg of the fraction A 1 a, dissolved in 2.5 ml acetic acid solution, were dialysed against 50 ml acetic acid solution of the same concentration in Visking cellulose tubing 8/32. The dialysis was carried out under magnetic stirring at 2° C during 24 hours and at different concentrations of acetic acid. Afterwards the outer solutions were lyophilized and assayed for oxytocic activity.

d) 25 mg of the fraction A 1, dissolved in 2.5 ml acetic acid solution, were dialysed against 50 ml acetic acid solution of the same concentration in Visking cellulose tubing 8/32. The dialysis was carried out under magnetic stirring at 2° C during 24 hours and at different concentrations of acetic acid. Afterwards the outer solutions were lyophilized and assayed for oxytocic activity.

chromatographic separation over Dowex 1X2

Dowex 1X2, 200–400 mesh, was purchased in the $Cl^{\ominus}$ form. The affinity of this strong basic anion exchanger is for $Cl^{\ominus} > CH_3COO^{\ominus} > OH^{\ominus}$. Complete substitution of $Cl^{\ominus}$ ions by $OH^{\ominus}$ ions respectively by $CH_3COO^{\ominus}$ ions is impossible with 1 N NH_4OH respectively 1 N acetic acid [46]. The conversion in the acetate form, compare [52], was carried out in a column by washing with water, 1 N NaOH, water, 1 N acetic acid and water. The column was then washed with 1 N sodium acetate until the pH of the effluent was 8.0–8.5. After rinsing with water, the column was washed with a 10 % sym. collidine–10 % pyridine buffer adjusted with acetic acid to pH = 9.0, until the pH of the effluent was also 9.0. Finally a treatment with starting-buffer, consisting of 1 % sym. collidine–1 % pyridine, adjusted with acetic acid to pH = 9.0, followed.

The filling of the 150 × 2 cm column, which was thermostated at 5° ± 0.2° C, was carried out with a suspension of the wet resin in an

equal volume of starting buffer, cooled to 5° C. The suspension was brought into the column in 5 portions; after settling of each portion the top 5 cm of the resin were stirred up with about 30 ml of the clear supernatant immediately before adding the next portion.

The fraction A 1 b was chromatographed in three separate portions of 320 mg each. The 320 mg A 1 b were suspended in 4 ml 1 % sym. collidine–1 % pyridine and centrifuged after 1 hour magnetic stirring. The residue was extracted twice in the same way, using 2 ml 1 % sym. collidine–1 % pyridine for each extraction, all at 4° C. The final residue A 1 b o was lyophilized. The yellow-brown extracts from 320 mg were taken together and, after being brought on the column, washed down with 2 ml 1 % sym. collidine–1 % pyridine and twice with 2 ml starting buffer. The column was then connected with a 2 L jar P, the contents of which could be stirred magnetically. The jar P was again connected with a 1 L jar Q, respectively a 2 L jar R. The elution scheme was:

1) 750 ml starting-buffer in P;

2) 1500 ml starting-buffer in P–750 ml 0.1 N acetic acid in Q;

3) 400 ml of the remaining mixture in P–400 ml 1.0 N acetic acid in R;

4) 100 ml of the remaining mixture in P–100 ml glacial acetic acid in R;

5) 400 ml glacial acetic acid in P.

The elution rate initially was 50 ml/hr, slowly diminishing to about 25 ml/hr at elution 5). Fractions of about 5 ml were collected. After leaving the column all fractions were mixed with 10 μl of glacial acetic acid within one hour and kept at 4° C. From all even fractions 250 μl was pipetted off, mixed with 2.0 ml ninhydrin reagent [35] and after colour development diluted with 4.2 ml 96 % ethanol : water = 1 : 1; the absorbancy at 570 mμ was measured. From all odd fractions also 250 μl was pipetted off, but these were subjected to alkaline hydrolysis with 0.5 ml 2.5 N NaOH during 2½ hours at 90–95° C. After cooling off to room temperature, the samples were neutralised with 0.5 ml 30 % acetic acid and developed with 2.0 ml ninhydrin reagent. After dilution with 3.5 ml 96 % ethanol : H_2O = 1 : 1, the absorbancy at 570 mμ was measured (fig. 3). The tubes belonging to a peak were taken together and the collected contents lyophilized.

paperelectrophoretic and paperchromatographic examination of the fractions A 1 b 1 – A 1 b 14

Paper electrophoresis was carried out on 57 × 12 cm strips of Whatman 3 MM in a pyridine/acetic acid/water = 100 : 4 : 900 (v/v/v) buffer of pH = 6.5. At 2000 Volt and 15–35 mA the electrophoresis took 1½ hours.

Paper chromatography was carried out at room temperature on 57 × 15 cm strips of Whatman 3 MM, descending, during 16–17 hours. The solvent systems used were:

I: n-butanol/acetic acid/pyridine/water = 30 : 6 : 20 : 24 (v/v/v/v); the solvent was always prepared 8 hours in advance.

II: n-butanol/acetic acid/water = 4 : 1 : 5 (v/v/v); the solvent was always prepared 8 hours in advance and immediately before use the upper (mobile) phase was separated. The lower phase in the chromatography jar was refreshed every 3 months.

The elution of the fractions separated on Whatman 3 MM was performed with 1 % acetic acid according to SANGER and TUPPY [47]. Total acid hydrolysis was carried out with twice-glass-distilled, constant-boiling hydrochloric acid (ca. 5.7 N) in fused jena glass capillaries at 110° C during 18 hours. The detection on paper was done with a collidine-ninhydrin reagent [29] or a cupric nitrate-ninhydrin reagent [36], which reagents, by virtue of their specific colouration with different amino acids, were a great help with the identifications. Besides, specific colour reactions for arginine [1], tyrosine [1] and tryptophane [53] were applied. Different modifications of the chlorination reaction [13, 20, 37, 61] were tried. This reaction was little used, however, as the reproducibility was rather poor. The best results were obtained with tert. butylhypochlorite as chlorinating agent [37].

RESULTS AND DISCUSSION

extraction and fractionation of fraction A on Sephadex G 25

The results of the extraction are represented in table 3. The number of units/mg of oxytocin in the starting material is somewhat higher than that of vasopressin. The fractionation scheme is summarised in table 1.

The result of the fractionation of fraction A is represented in fig. 1. It is in accordance with the curve obtained by PORATH *et al.* [33]. The reproducibility in 8 separate fractionations was very good.

We estimated the biological activities of the fractions Al and A 2 in some of the 8 fractionations. It was found that the biological activity of the high-molecular fraction A 1 increased at the expense of the biological activity of the low-molecular fraction A 2 when going from the 1st to the 8th fractionation (table 2). Apparently a re-association of partly dissociated "van Dyke's protein" is taking place when the solution A is stored (about 1 month at 4° C). Compare also [33].

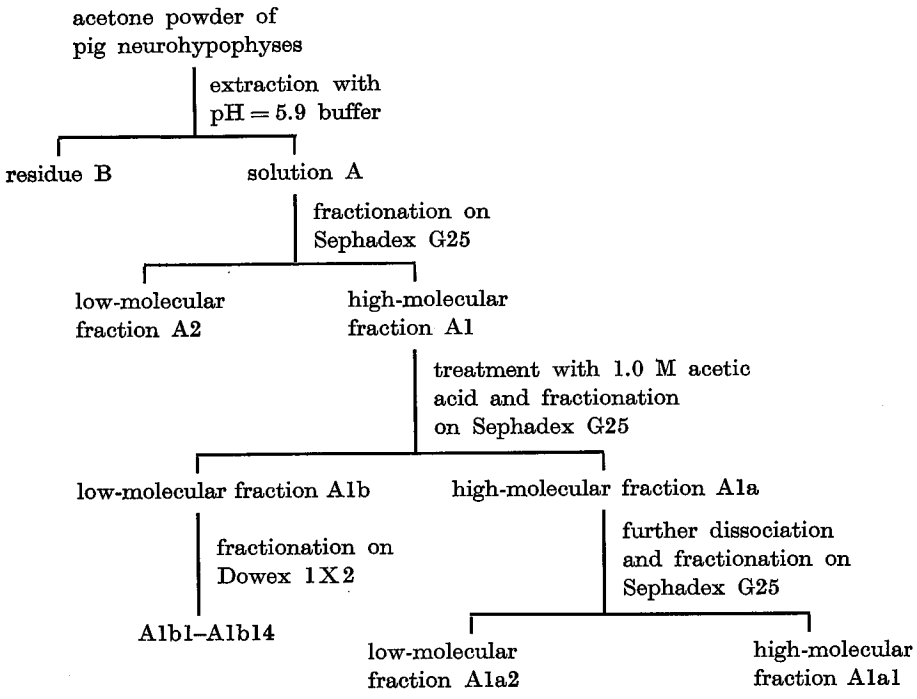
dissociation and fractionation of fraction A 1

PORATH *et al.* [33] effected the dissociation of van Dyke's protein with 1 M formic acid at 70° C during 10 minutes. Former observations have shown that this dissociation could also take place under very mild conditions, for example by dialysis against 0.2 N acetic acid. It seemed likely that 1 N acetic acid should lead to dissociation.

After treatment of A 1 with 1.0 N acetic acid, fractionation was

TABLE 1

Fractionation scheme and survey of the fraction codifications.



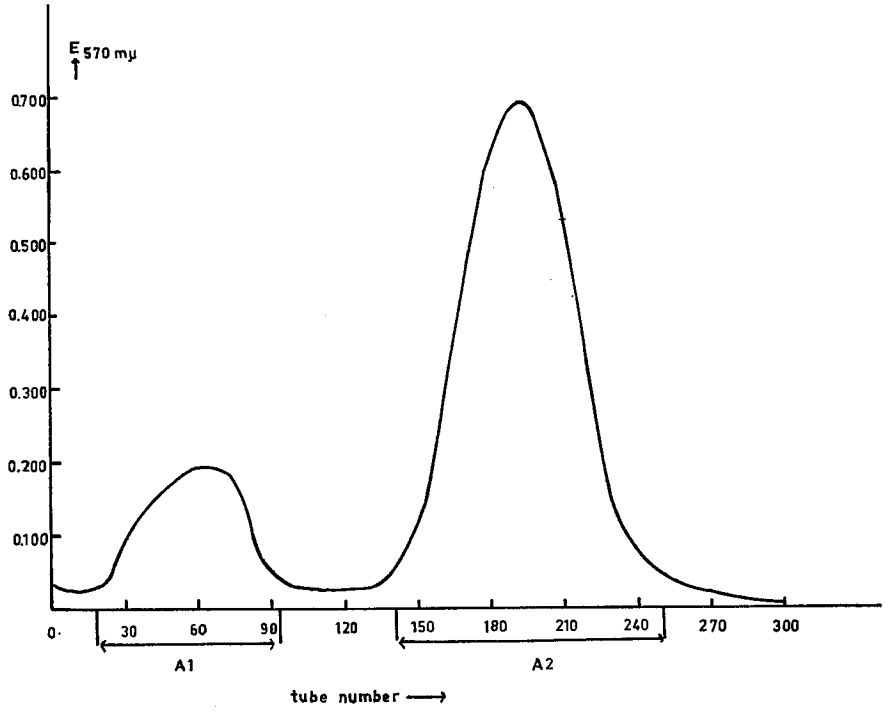


Fig. 1. Gel filtration of solution A over Sephadex G25

TABLE 2

Oxytocic and pressor activity over the fractions A1 and A2 in the first and in the last fractionation of the extract A. When the last (8th) fractionation took place the extract had been stored 1 month at 4°C.

fraction	oxy units/mg	pressor units/mg	fraction	oxy units/mg	pressor units/mg
A1 1st fractionation	6.0	3.8	A2 1st fractionation	1.0	1.15
A1 8th fractionation	8.1	4.5	A2 8th fractionation	0.75	0.55

performed with Sephadex G 25 (fig. 2). The reproducibility was good. The curve deviates somewhat from the curve described by PORATH *et al.* [33]; a distinct intermediate fraction is missing. This can be explained by the fact that different dissociation conditions were used.

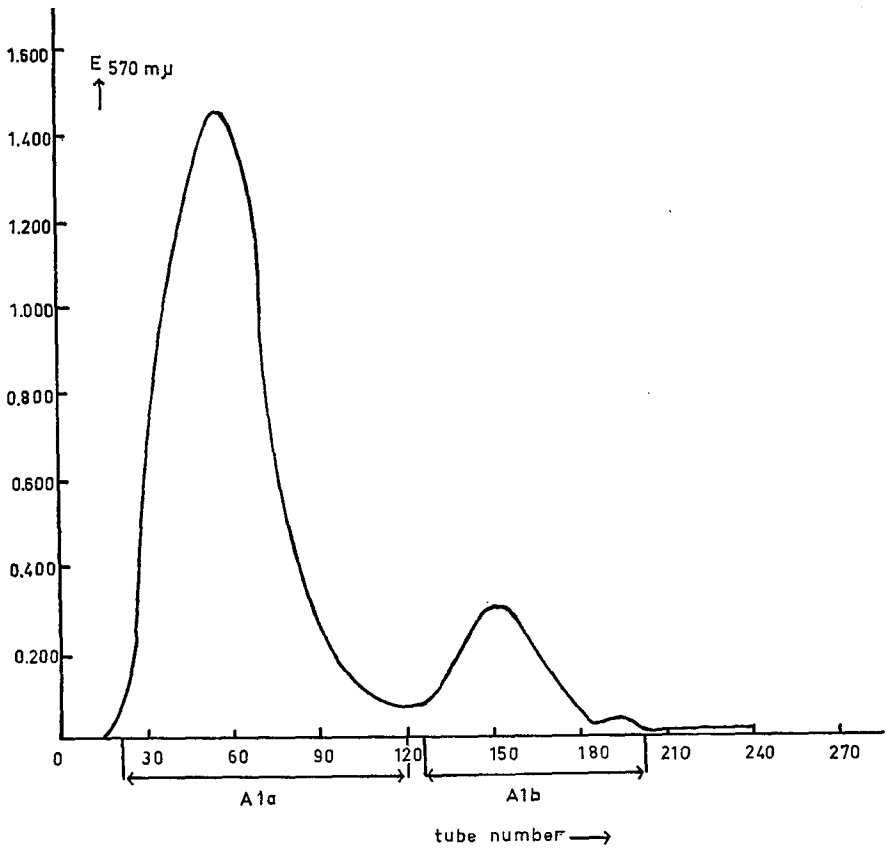


Fig. 2. Gel filtration of fraction A1 after treatment with 1.0 N acetic acid over Sephadex G25.

The total yield in the fractions A1a and A1b is for lysine-vasopressin 60.800 pressor units (49 %) and for oxytocin 125.800 oxytocic units (73.4 %). In the low-molecular fraction A1b the ratio pressor activity : oxytocic activity = 1 : 1. The biological activity of the high-molecular

fraction A1a can be attributed wholly to oxytocin; the pressor activity in this fraction can be explained by the intrinsic pressor activity of oxytocin [14]. It is remarkable, however, that there is still oxytocic activity in the high-molecular fraction A1a.

dissociation of the fractions A1a and A1

In order to investigate whether this oxytocic activity of A1a is due to incompletely liberated oxytocin or to another oxytocic principle, we examined whether the oxytocic activity was dissociable. Indeed, A1a could be split into a high-molecular fraction A1a1(A1a1′) and a low-molecular fraction A1a2(A1a2′). The results are summarized in the tables 3a and b.

TABLE 3

Dissociation of fraction A1a

a) treatment of A1a with 1 M formic acid, followed by gel filtration over Sephadex G25

fraction	weight	weight in %	oxy *U*/mg	oxy *U* total	oxy *U* in %
A1a	500 mg	100	3.3	1650	100
A1a1	429.3 mg	85.9	0.6	257	15.6
A1a2	10.1 mg	2.0	132.4	1337	81.0

b) prolonged treatment of A1a with 1 M acetic acid, followed by gel filtration over Sephadex G25.

fraction	weight	weight in %	oxy *U*/mg	oxy *U* total	oxy *U* in %
A1a	200 mg	100	3.3	660	100
A1a1′	186.8 mg	93.4	0	0	—
A1a2′	— *)	—	—	605	91.6

*) Because A1a2′ was highly hygroscopic, it was not possible to determine its weight accurately.

In the low-molecular fraction A1a2, which contains practically all oxytocic activity, a number of ninhydrin-positive substances are present. One of these, which is indistinguishable from oxytocin in paperelectrophoresis and paperchromatography, gave upon total acid hydrolysis exclusively the amino acids of oxytocin. Also in the biological test, this fraction was found to be indistinguishable from oxytocin; all other components were devoid of oxytocic activity. We conclude, therefore, that the oxytocic activity of A1a is caused by normal oxytocin, which apparently was not completely liberated during the first treatment with 1.0 M acetic acid.

In order to compare the behaviour of the original fraction A 1 with that of A1a, both were dialysed against different acetic acid concentrations (see tables 4a and 4b).

TABLE 4

a) dialysis of A1a against different concentrations of acetic acid

acetic acid concentration	pH	outer solution		
		oxy *U*/ml	oxy *U* total	oxy *U* in %
0.1 N	2.87	0.56	28.0	34
0.5 N	2.48	0.73	36.5	44
1.0 N	2.30	0.83	41.5	50
2.5 N	2.01	0.96	48.0	58
5.0 N	1.37	1.04	52.0	63

b) dialysis of A1 against different concentrations of acetic acid

acetic acid concentration	pH	outer solution		
		oxy *U*/ml	oxy *U* total	oxy *U* in %
0.1 N	2.87	1.15	57.5	32
0.5 N	2.48	1.66	83.0	47
1.0 N	2.30	1.75	87.5	49
2.5 N	2.01	2.07	103.5	58
5.0 N	1.37	2.20	110.0	62

Both fractions behaved similarly. Obviously a dissociation–equilibrium exists, which in 1M acetic acid is shifted to complete liberation of oxytocin. With increasing concentrations of acetic acid also oxytocin is liberated completely.

In this connection it is interesting that DU VIGNEAUD *et al.* [55] showed, that desamino-oxytocin, in contrast to oxytocin, does not form a complex with neurophysin.

fractionation of A1b over Dowex 1X2

Fig. 3 shows the separation of the amino acids and peptides present in A1b.

Table 5 gives a survey of the weight percentages and biological activities during the whole fractionation procedure. The total yield in weight of the fractions A1b1–A1b14 separated on Dowex 1X2 is 775 mg = 81 %, the weight of A1b taken as 100 %. The distribution of this percentage is as follows: ca. 10 % in the residue A1bo, ca. 26 % in the vasopressin (A1b1) and oxytocin (A1b4–6) fractions, ca. 25 % in the acidic (A1b13–14) fractions and the remaining 20 % divided over a number of smaller fractions.

The yield in biological activity after the fractionation on Dowex 1X2 is for lysine-vasopressin 86 %, for oxytocin 40 %, the biological activity of A1b taken as 100 %. Thus, a considerable loss in oxytocic activity takes place in this step. It is possible that the high pH of the starting-buffer plays a role in this loss of oxytocic activity [12]. Generally, the

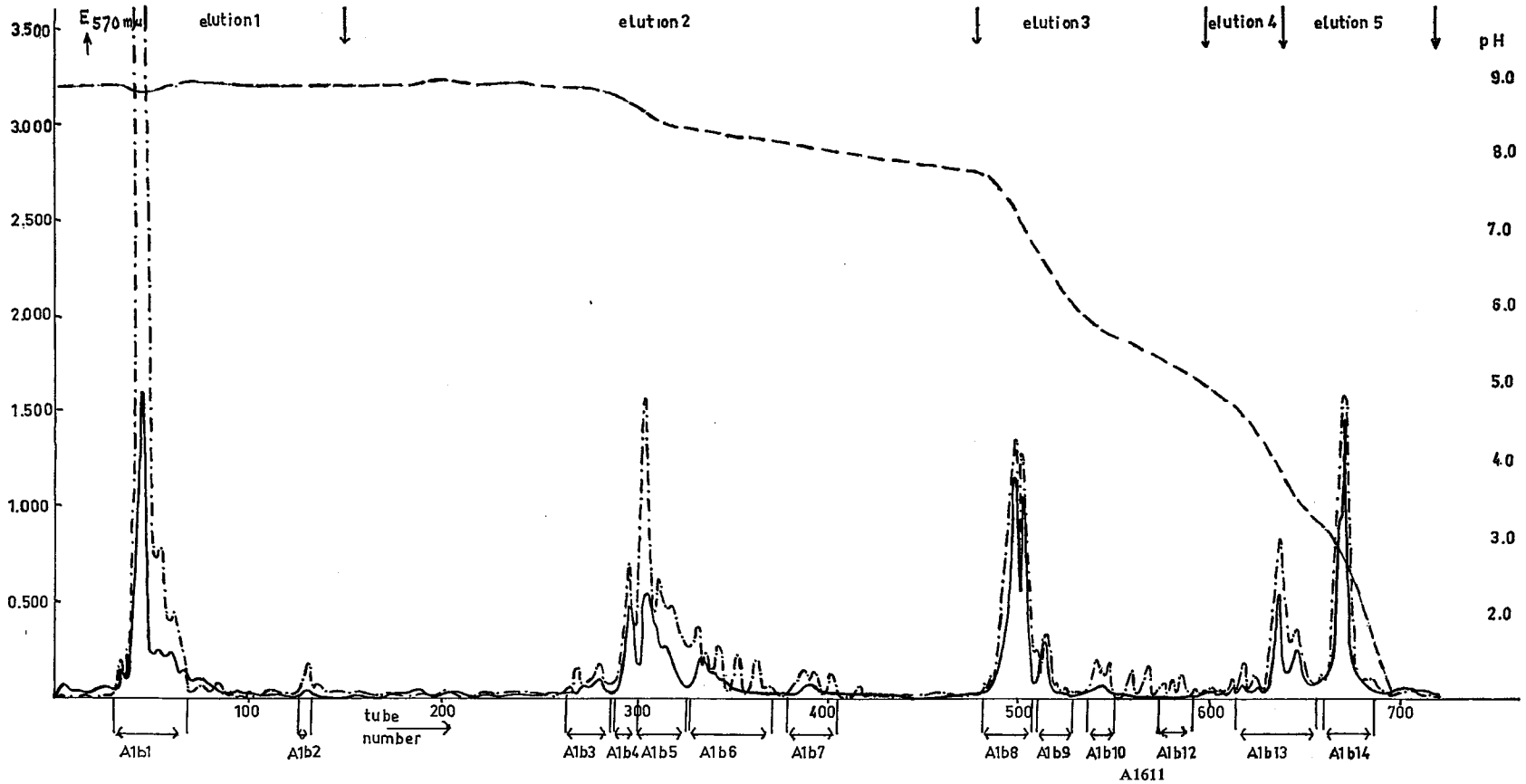


Fig. 3. Fractionation of fraction A1b over Dowex 1X2 ——— ninhydrin colour without hydrolysis

-·-·-·-·- ninhydrin colour after alkaline hydrolysis - - - - - pH of the effluent during elution

TABLE 5

Biological activity and weight of the different fractions. All yields in % are calculated on the basis of acetone-desiccated posterior lobe powder. For A1 a mean value for the biological activity was taken (cf. table 2). – means: not determined.

Fraction	weight in mg	weight in %	oxy *U*/mg	oxy *U* total	oxy *U* in %	pressor *U*/mg	pressor *U* total	pressor *U* in %
posterior lobe acetone powder	95300	100	1.8	171500	100	1.3	123890	100
residue B	43540	46	0.08	3500	3.0	0.05	2200	1.8
high-mol. fr. A1	23940	25.1	7.1	170000	99	4.2	100800	81.4
low-mol. fr. A2	14740	15.2	0.85	12300	7.2	0.85	12300	9.9
high-mol. fr. A1a	20530	21.5	3.3	66700	38.9	0.04	800	0.6
low-mol. fr. A1b	960	1.0	61.6	59100	34.5	62.5	60000	48.4
residue A1bo	96	0.1	0.7	70	—	5.8	550	0.4
Dowex fr. A1b1	250	0.26	4.8	1200	0.7	200	50000	40.4
A1b2	11	0.01	—	—	—	75	825	0.7
A1b4	16	0.02	105	1680	1.0	—	—	—
A1b5	60	0.06	310	18600	10.9	4.8	288	0.2
A1b6	28	0.03	60	1680	1.0	—	—	—
A1b8	29	0.03	—	—	—	—	—	—
A1b9	17	0.02	—	—	—	—	—	—
A1b13	81	0.09	—	—	—	—	—	—
A1b14	163	0.17	—	—	—	—	—	—

yield of oxytocin is lower than that of vasopressin [7, 32]. The overall yield in biological activity amounts to 40.4 % for nearly pure lysine-vasopressin, which is comparable with the overall yield reported for arginine-vasopressin [32]. For considerably purified oxytocin the overall yield amounts to 13 %. However, 38.9 % of the original oxytocic activity is still present in fraction A1a.

paper-electrophoretic and -chromatographic examination of the fractions A1b1–A1b14

The electropherogram of the fractions A1b1 and A1b5 is given in fig. 4.

The R_F values of the subfractions and the results of the total acid hydrolyses are summarised in table 6. The most important impurities in the lysine-vasopressin fraction are free lysine and free arginine. Lysine-vasopressin was also found in the fractions A1b2 and A1b3. The high amount of free proline in fraction A1b2 is remarkable. Oxytocin too is distributed over three fractions, namely A1b4–6. The amount of impurities, however, is considerably greater than in the case of vasopressin. The main fraction A1b5 contains, apart from oxytocin, free glutamic acid and free valine, the fractions A1b4 and A1b6 contain free asparagine and free serine, together with some peptides, and the compounds A, B and C. The nature of these three compounds was not investigated.

The remaining neutral and acidic fractions consist, apart from free

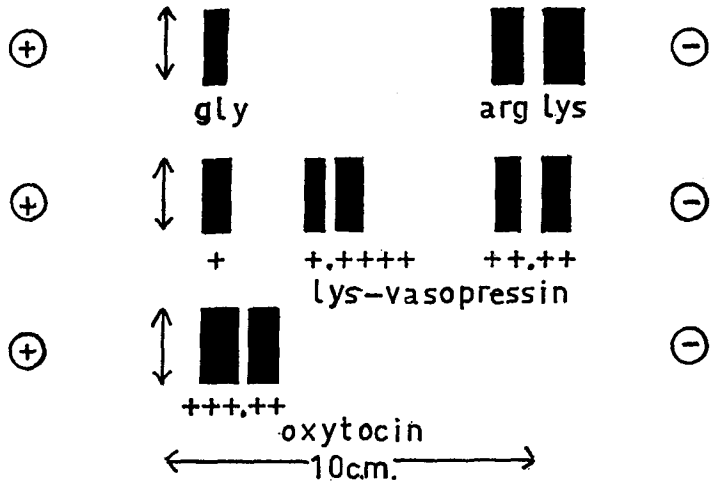


Fig. 4. Electropherogram of respectively the reference amino acids glycine, arginine and lysine, the fraction A1b1 and the fraction A1b5 (from the top downwards). The starting position is marked by ↕. The relative intensities are denoted by one or more + signs.

phenylalanine and leucine, exclusively of peptides. Their number is considerable, and they can be arranged in groups which seem to be closely related. In accordance with the data of WINNICK *et al.* [44, 62], the most abundant amino acids present in these peptides are alanine, glutamic acid, glycine, aspartic acid, leucine and valine. Also proline and, to a lesser degree, serine, tyrosine and phenylalanine, were frequently found in the acid hydrolysates. It is not yet clear whether these peptides have any biological significance.

ACKNOWLEDGEMENT

The authors wish to thank Miss W. Klaver, Mr. P. S. Balogh and Mr. D. G. de Rooy for their skilful assistance.

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TABLE 6

R_F values and amino compositions of the subfractions obtained after paper chromatography on Whatman 3 MM paper of the fractions A1b1–A1b14. Fractions giving a faint colour reaction with ninhydrin are generally not tabulated.

Fraction	R_F in I	R_F in II	Relative intensity	Colour reaction with ninhydrin[52]	Amino acids obtained after total acid hydrolysis																	Conclusion
					$(cys)_2$	lys	arg	asp	glu	gly	ser	ala	pro	val	met	tyr	leu	ileu	phe	A	B	C	
A1b1a	0.18	0.08	++	purple		lys																	lys
A1b1b	0.23	0.12	+±	,,			arg																arg
A1b1c	0.36	—	+	,,																			—
A1b1d	0.48	0.19	++++±	yellow → purple	$(cys)_2$	lys		asp	glu	gly			pro			tyr			phe				lys-vasopressin[1]
A1b1e	0.56	—	+	purple																			—
A1b2a	0.18	0.08	++	purple		lys																	lys
A1b2b	0.23	0.12	++	,,			arg																arg
A1b2c	0.34	0.29	++++±	yellow									pro										pro
A1b2d	0.48	0.19	+++	yellow → purple	$(cys)_2$	lys		asp	glu	gly			pro			tyr			phe				lys-vasopressin[1]
A1b3a	0.30	0.25	+++±	redviolet																A[2]			A[2]
A1b3b	0.48	0.19	+±	yellow → purple	$(cys)_2$	lys		asp	glu	gly			pro			tyr			phe				lys-vasopressin[1]
A1b4a	0.30	0.25	++++	redviolet																A[2]			A[2]
A1b4b	0.47	0.44	+	purple																			—
A1b4c	0.59	0.60	++	,,																	B[3]		B[3]
A1b4d	0.70	0.40	++	yellow → blue	$(cys)_2$			asp	glu	gly			pro			tyr	leu	ileu					oxytocin[4]
A1b5a	0.22	0.09	++	purple																		C[5]	C[5]
A1b5b	0.24	0.16	++	brown						gly													gly
A1b5c	0.30	0.25	+±	redviolet																A[2]			A[2]
A1b5d	0.45	0.44	+++	purple										val									val
A1b5e	0.59	0.60	++++	,,																	B[3]		B[3]
A1b5f	0.70	0.40	+++	yellow → blue	$(cys)_2$			asp	glu	gly			pro			tyr	leu	ileu					oxytocin[4]
A1b6a	0.18	0.08	++±	orange				asp															asp (NH_2)
A1b6b	0.22	0.12	+++	purple					glu		ser											C[5]?	peptide/mixture
A1b6c	0.25	0.13	++++	greyblue							ser!				met								Ser + peptide
A1b6d	0.30	—	+++	redviolet																A[2]			A[2]
A1b6e	0.46	0.44	+±	purple		lys		asp	glu	gly				val!									val + peptide
A1b6f	0.50	0.43	+++	,,											met								met
A1b6g	0.58	0.60	+++	,,																	B[3]		B[3]
A1b6h	0.70	0.38	++	yellow → blue	$(cys)_2$			asp	glu	gly			pro			tyr	leu	ileu					oxytocin[4]
A1b7a	0.21	—	++	purple				asp	glu	gly		ala										C[5]	peptide (+ C[5])?
A1b7b	0.25	—	+±	,,					glu	gly	ser												peptide
A1b7c	0.31	—	+±	redviolet				asp	glu	gly										A[2]			peptide (+ A[2])?
A1b7d	0.46	—	++	purple				asp	glu	gly				val									peptide
A1b7e	0.56	0.53	++++	brownviolet															phe				phe
A1b7f	0.59	0.60	+++	purple													leu						leu
A1b8a	0.42	—	+	yellow → violet				asp	glu	gly		ala	pro	val			leu						peptide

A1b9b	0.20	–	+±	redviolet				asp	glu	gly	ser?	ala											peptide
A1b9c	0.25	–	++	purple				asp				ala	pro							A²)?			peptide
A1b9d	0.31	–	++	redviolet		lys?		asp	glu	gly	ser	ala		val						A²)			peptide (+ A²)?)
A1b9e	0.37	–	+±	yellow → violet		lys?		asp	glu	gly		ala	pro?	val									peptide
A1b9f	0.43	–	+++	purple	$(cys)_2$	lys		asp	glu	gly		ala?	pro	val?		tyr!	leu?		phe				peptide
A1b9g	0.47	–	++	„		lys?		asp	glu	gly	ser	ala	pro	val		tyr	leu		phe				peptide
A1b9h	0.50	–	+++±	„	$(cys)_2$	lys		asp	glu	gly	ser?	ala	pro	val		tyr	leu		phe				peptide
A1b9i	0.53	–	++	„		lys!		asp	glu	gly	ser?	ala		val			leu						peptide
A1b9j	0.56	–	++	yellow → violet									pro!				leu?						peptide
A1b9k	0.63	–	+±	„									pro				leu						peptide
A1b9l	0.76	–	++	purple								ala		val			leu						peptide
A1b9m	0.79	–	+++	„								ala		val			leu						peptide
A1b9n	0.84	–	+±	„										val			leu!						peptide
A1b9p	0.86	–	+	yellow → violet									pro				leu						peptide
A1b10a	0.51	0.52	+++	purple				asp	glu	gly	ser!		pro						phe!				peptide
A1b11a	0.52	–	+±	purple				asp	glu	gly	ser		pro						phe				peptide
A1b11b	0.86	0.87	++±	„													leu						peptide
A1b12a	0.49	–	+±	purple		lys		asp	glu	gly			pro			tyr			phe				peptide
A1b12b	0.87	–	+	violet													leu						peptide
A1b13a	0.22	0.18	+++±	purple					glu			ala											peptide
A1b13b	0.28	–	++	„					glu	gly			pro							A²)			peptide (+ A²)?)
A1b13c	0.39	–	+±	„				asp	glu			ala		val						A²)			peptide
A1b13d	0.47	–	+	„				asp	glu	gly		ala	pro	val		tyr	leu			A²)			peptide
A1b13e	0.50	–	++±	„					glu			ala	pro				leu		phe				peptide
A1b13f	0.68	–	+±	„				asp?	glu				pro?				leu		phe				peptide
A1b14a	0.16	0.14	+++±	bluegreen				asp!	glu!	gly		ala	pro?										peptide
A1b14b	0.20	0.16	+++	purple				asp	glu!	gly		ala	pro										peptide
A1b14c	0.22	0.22	+++	„				asp	glu!			ala											peptide
A1b14d	0.26	0.27	++++	greyviolet				asp	glu!			ala!											peptide
A1b14e	0.41	0.18	++	purple				asp!	glu	gly		ala	pro	val?		tyr	leu		phe				peptide
A1b14f	0.52	0.59	++	„				asp	glu	gly		ala	pro	val?		tyr?	leu		phe				peptide

[1]) The R_F values obtained for lysine vasopressin differ somewhat from those mentioned in the literature (for Whatman 1): 0.87 (I) [4, 8, 21]), 0.56 (I) [12])
0.65 (II) [4, 8, 21, 38]), 0.28? (II) [12])

[2]) The compound A was not further identified. The R_F values for A differ only slightly from those for alanine, but there is a striking difference in colour after reaction with ninhydrin. Identity of A with β-alanine was excluded. The compound A was recovered unchanged after treatment with 6 N hydrochloric acid.

[3]) The compound B was not further identified. The typical feature for this compound is, that after acid hydrolysis a positive colour reaction with ninhydrin was no longer obtained.

[4]) The R_F values obtained for oxytocin differ from those mentioned in the literature, (for Whatman 1): 0.26 (I) [8, 21]), 0.23 (I) [38])
0.08–0.11 (II) [8, 21]), 0.11–0.16 (II) [28]), 0.11? (II) [12]).

[5]) The compound C was not further identified. After acid hydrolysis the R_F values shift from 0.22 to 0.41 in I, and from 0.09 to 0.27 in II.

A very strong intensity is denoted by !

A doubtful identification is denoted by ?