Solubility of the Hypothalamic Hormones TSH-Releasing Factor (TRF) and LH-Releasing Factor (LRF) in Organic and Alcoholic Solvents

Recent evidence from this laboratory has been accumulating in favor of the concept that the hypothalamic hormone TRF (TSH-releasing factor) is not a simple polypeptide[1-4]. We have recently reported[4] on high resolution nuclear magnetic resonance (NMR) spectra of TRF; these spectra were obtained in D_2O. During the course of these studies it became necessary to investigate the possibility of using deuterated solvents other than D_2O to explore in more detail the complete resonance spectrum. As a prerequisite to the contemplated NMR studies, we were led to investigate the solubility of purified TRF in (non-deuterated) organic solvents. Results obtained with $CHCl_3$, methanol and pyridine suggested further studies on the solubility and extractability of TRF and LRF (LH-releasing factor) in these and related solvents, earlier reports in the literature having already shown the possibility of extracting LRF or TRF with phenol or glacial acetic acid[5-7] at various stages of purification.

It is the purpose of this note to report on these recent observations.

Materials and methods. (A) *Purified preparations of TRF and LRF.* For the studies on the solubility of TRF in organic solvents, we used a purified preparation with a specific activity of 80 U/mg[8], prepared through stage 3 of the purification sequence described in [1]. Solvents used were Fisher Certified Reagent grade. Aliquots containing 1.5 mg dry weight of the TRF preparation were extracted at 38 °C for 18 and 26 h respectively in 1.0 ml chloroform and pyridine, and in absolute methanol for 3 h at room temperature. Each mixture was then centrifuged in 1.0 ml at 2000 *g* for 5–15 min, the supernatant was drawn off and the residue was washed twice with 1 ml portion of solvent. The combined supernatants were concentrated to dryness in vacuo at 45 °C. Extracts and residues were redissolved in 0.9% saline for the bioassays. All bioassays were 4-point assays, with 2 doses (5 U and 15 U) of the TRF preparation used in the various extraction procedures vs 2 equivalent doses of extracts and residues. The assay for TRF is the current version of the in vivo method described previously[9], see also [10]. Partition between dichloromethane and water was studied using a preparation of highly purified TRF (15,000 U/mg)[3]; the bioassay in this case was conducted at 1 dose level only.

For studies on the solubility of LRF in organic solvents we used a purified preparation corresponding to stage 1 of the sequence described in [1]. Aliquots of 240 mg of this LRF preparation were extracted in 20 ml chloroform or

Table I. (a) Studies on solubility of purified TRF in chloroform, methanol, pyridine and partition between water/dichloromethane; (b) studies on the extraction of TRF from crude starting materials by absolute or 90% methanol

Protocol No.	Treatments	No. of animals	Adjusted mean ± S.E.	P
7865 (a)	Saline control	5	2.357 ± 0.014	
18-h	TRF S_1	5	2.428 ± 0.013	**
extractions	TRF S_2	5	2.483 ± 0.013	**
	$CHCl_3$ extract U_1	5	2.368 ± 0.013	–
	$CHCl_3$ extract U_2	5	2.435 ± 0.014	**
	Pyridine extract X_1	5	2.349 ± 0.013	–
	Pyridine extract X_2	5	2.358 ± 0.013	–
7876 (a)	Saline control	5	2.379 ± 0.019	
26-h	TRF S_1	5	2.527 ± 0.019	**
extractions	TRF S_2	5	2.589 ± 0.019	**
	$CHCl_3$ extract U_1	5	2.460 ± 0.019	*
	$CHCl_3$ extract U_2	5	2.558 ± 0.019	**
	$CHCl_3$ residue V_1	5	2.443 ± 0.019	–
	$CHCl_3$ residue V_2	5	2.520 ± 0.019	**
	Pyridine residue A_1[c]	5	2.500 ± 0.019	**
	Pyridine residue A_2	5	2.585 ± 0.019	**
7888 (a)	Saline control	5	2.303 ± 0.013	
	TRF S_1	5	2.368 ± 0.013	**
	TRF S_2	5	2.430 ± 0.013	**
	Absolute methanol extract A_1	5	2.357 ± 0.013	*
	Absolute methanol extract A_2	5	2.426 ± 0.013	**
	Absolute methanol residue B_1	5	2.306 ± 0.013	–
	Absolute methanol residue B_2	5	2.289 ± 0.013	–
	Pyridine residue C_1[d]	5	2.378 ± 0.013	**
	Pyridine residue C_2	5	2.423 ± 0.013	**
7409 (a)	Saline control	5	2.283 ± 0.017	
	TRF[e]	5	2.445 ± 0.017	**
	Dichloromethane phase[e]	5	2.313 ± 0.018	–
	Aqueous phase[e]	5	2.451 ± 0.017	**
7897 (b)	Saline control	5	2.219 ± 0.027	
	Absolute methanol extract A_1	5	2.288 ± 0.026	–
	Absolute methanol extract A_2	5	2.382 ± 0.026	**
	Absolute methanol extract boiled B_1	5	2.298 ± 0.026	–
	Absolute methanol extract boiled B_2	5	2.410 ± 0.029	**
	2*N* HOAc extract C_1	3	2.396 ± 0.033	**
	2*N* HOAc extract C_2	4	2.450 ± 0.030	**
	2*N* HOAc extract boiled D_1	4	2.310 ± 0.029	–
	2*N* HOAc extract boiled D_2	5	2.506 ± 0.026	**
7917 (b)	Saline control	5	2.190 ± 0.018	
	90% methanol extract U_1	5	2.281 ± 0.018	*
	90% methanol extract U_2	5	2.331 ± 0.018	**
	90% methanol residue X_1	5	2.245 ± 0.018	–
	90% methanol residue X_2	5	2.255 ± 0.019	–
	Absolute methanol residue P_1[f]	5	2.272 ± 0.018	*
	Absolute methanol residue P_2	5	2.363 ± 0.018	**

Adjusted mean ± S.E. = abridged $\log_{10}$ of the mean value of the radioactivity (c/min) measured in the 2nd blood sample after covariance adjustment to radioactivity of 1st blood sample, calculated as in [13]. S.E., standard error. P, probability of statistical difference when compared to the control group by the multiple comparison test of DUNNETT[13]; –, not significant; * 0.05 level; ** 0.01 level. Experimental treatments administered at 2 dose-levels with equal geometric intervals in each experiment, for calculating potency ratios in 4-point assays (not reported here); the subscripts $_1$ and $_2$ (such as in S_1, S_2) refer respectively to the low and high dose; [c] residue from the 18-h pyridine extraction from experiment 7865; [d] residue from the 26-h pyridine extraction from experiment 7876; [e] treatments at 1 dose level (0.5 µg TRF, 15,000 U/mg); [f] residue from the absolute methanol extraction from experiment 7897.

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methanol at room temperature for 2 h. After centrifugation at 2000 *g* for 10 min, extracts and residues were dried and redissolved in 0.01 *N* acetic acid in physiological saline for the bioassays. The assay utilized to assess LRF activity is the ovarian ascorbic acid depletion method modified from PARLOW and calculated as in [11]; the 2 ovaries are removed 3 h apart and the ascorbic acid content of the second ovary is adjusted by covariance to the ascorbic acid content of the first ovary.

(B) *Crude preparations of TRF and LRF.* For the studies on extractability of TRF and LRF from crude starting material, the 2 *N* acetic acid extract of the acetone powder of 2,000 sheep hypothalamic fragments prepared as in [1] was used. The extraction was carried out at room temperature for 12 h, using 100 ml of chloroform, absolute methanol or aqueous methanol (90%) for 10 g of dry powder. The bioassays for TRF and LRF were similar to those described above in (A); LRF and TRF activities were assayed at doses corresponding to 2 and 6 hypothalamic fragments/assay animal.

All calculations for all bioassays reported here are handled by electronic computers (IBM 7094) using the program EXBIOL written by SAKIZ or its modification EXBIOL in the case of LRF assays.

Results and discussion. It can be seen from the results presented in Table I that purified TRF (80 U/mg) is partially soluble in chloroform, highly soluble in absolute methanol and completely insoluble in pyridine. Other results in Table I show that TRF activity can be extracted by absolute methanol from the crude 2 *N* acetic acid extract of the acetone powder of sheep hypothalamic fragments. The absence of significant difference between the results obtained with boiled (15 min in boiling water) vs non-boiled extract would indicate that little, if any, TSH is present in the extracts at the relatively small quantities administered, as it would be expected that boiling would inactivate TSH on the basis of many data available in the literature and confirmed in this laboratory. Table I shows also that highly purified TRF is not extractable from water by dichloromethane.

The results presented in Table II show that LRF is not soluble in chloroform (all the activity being recovered in the residue) and that it is highly soluble in absolute methanol. On the other hand, absolute methanol does not appear to be a choice solvent to extract LRF from crude starting material; aqueous methanol (90%) being much more efficient in this respect.

In the past we have used aqueous methanol extraction for CRF from crude hypothalamic extracts[12] and also from crude posterior pituitary extract. The results reported here show that solubility in organic or alcoholic solvents may also be used to advantage at some stage of the purification of TRF and LRF[14].

Table II. (a) Solubility of purified LRF in chloroform, absolute methanol and 90% methanol; (b) extraction of LRF from crude starting material by absolute or 90% methanol

Protocol No.	Treatments	No. of animals	Adjusted mean ± S.E.	P
7868 (a)	Saline control	5	150.76 ± 3.16	
	LRF control	5	116.80 ± 3.15	**
	$CHCl_3$ extract X_1	5	145.45 ± 3.18	–
	$CHCl_3$ extract X_2	5	143.10 ± 3.15	–
	$CHCl_3$ residue A_1	5	120.12 ± 3.16	**
	$CHCl_3$ residue A_2	5	112.01 ± 3.26	**
7896 (a)	Saline control	5	160.00 ± 4.41	
	LRF control	5	132.34 ± 4.48	**
	Absolute methanol extract A_1	5	124.09 ± 4.42	**
	Absolute methanol extract A_2	5	112.71 ± 4.42	**
	Absolute methanol residue B_1	5	151.25 ± 4.45	–
	Absolute methanol residue B_2	5	154.06 ± 4.45	–
7904 (b)	Saline control	5	143.52 ± 6.05	
	Absolute methanol extract X_1	5	135.37 ± 6.00	–
	Absolute methanol extract X_2	5	123.91 ± 6.22	*
	Absolute methanol extract (boiled) U_1	5	136.17 ± 6.07	–
	Absolute methanol extract (boiled) U_2	5	122.34 ± 6.12	*
	2 *N* HOAc extract P_1	5	93.51 ± 6.02	**
	2 *N* HOAc extract P_2	5	98.62 ± 6.08	**
	2 *N* HOAc extract (boiled) Q_1	5	121.54 ± 6.00	*
	2 *N* HOAc extract (boiled) Q_2	5	99.31 ± 6.00	**
7915 (b)	Saline control	5	137.08 ± 5.13	
	90% methanol extract U_1	4	105.12 ± 5.74	**
	90% methanol extract U_2	5	103.05 ± 5.13	**
	90% methanol extract (boiled) A_1	3	111.38 ± 7.19	*
	90% methanol extract (boiled) A_2	2	108.77 ± 8.66	*
	90% methanol residue B_1	5	133.12 ± 5.17	–
	90% methanol residue B_2	5	115.81 ± 5.13	*
	90% methanol residue (boiled) C_1	5	123.06 ± 5.20	–
	90% methanol residue (boiled) C_2	4	127.36 ± 6.10	–

Adjusted mean ± S.E. = mean of ovarian ascorbic acid content (in μg ascorbic acid) of second ovary adjusted by covariance to the ascorbic acid content of first ovary (see [10]). S.E., standard error. P, probability of statistical significance when compared to the saline control group by the multiple comparison test of DUNNETT; –, not significant; *, 0.05 level; **, 0.01 level. Explanations for subscripts as in Table I.

Résumé. L'hormone hypothalamique TRF (TSH-releasing factor) est soluble dans l'alcool méthylique à 90%, partiellement soluble dans l'alcool méthylique absolu ou le chloroforme; TRF n'est pas soluble dans la pyridine. Le chlorure de méthylène (dichlorométhane) n'extrait pas le TRF d'une solution aqueuse (préparation hautement purifiée, 15000 U/mg). L'hormone hypothalamique LRF (LH-releasing factor) n'est pas soluble dans le chloroforme mais est soluble dans l'alcool méthylique absolu ou à 90%.

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