A Correlation of Release of Polypeptide Hormones and of Immunoreactive Neurophysin from Isolated Rat Neurohypophyses

The nature of the cellular mechanisms involved in the release of polypeptide hormones from the neurohypophysis following the influx of calcium into the depolarized nerve endings[1] has not yet been fully characterized. Some investigators[2] have suggested that secretion occurs from an extragranular pool; on the other hand, DOUGLAS et al.[1,3] have proposed that release may occur by exocytosis (reverse pinocytosis, emiocytosis), and have recently published electron micrographs that show images consistent with exocytosis in rat and hamster neurohypophyses[4]. They have suggested that a critical test to differentiate between the 2 hypotheses would be to determine whether neurophysins are released with the polypeptide hormones upon stimulation.

Neurophysins are sulfur-rich proteins, whose subcellular distribution is similar to that of oxytocin and vasopressin[5,6]. They represent as much as 50% of the soluble proteins found in the neurosecretory granules[2]. Thus release of such proteins in response to the same stimuli inducing polypeptide hormone release would be more consistent with the exocytosis hypothesis.

With the advent of a specific and sensitive radioimmunoassay for neurophysins[7–9], it has been demonstrated that plasma neurophysin levels rise in conditions presumed to be associated with increased secretion of neurohypophysial hormones in man[7] and in experimental animals[10]. The present study has employed an in vitro preparation allowing for determination of polypeptide hormone and neurophysin release.

The neurohypophysis was isolated from each of 54 rats (200–300 g body wt.) which had free access to water and food to the time of decapitation. The cut end of the pituitary stalk was tied to a platinum wire and immersed in 2.0 ml of Locke's solution in a test tube continuously gassed with 95% O_2 – 5% CO_2. After a 10 min preincubation period, the preparation was transferred into a solution in which 154 m*M* NaCl had been replaced by an equimolar amount of choline chloride, since it has been demonstrated that release of neurohypophysial hormones is potentiated in sodium-deficient media[1,11]. Secretion was evoked by electrical stimuli (30 sec train, 2 msec bipolar pulses, 0.25 to 10.0 mA, 50 Hz) applied as described elsewhere[12], and was followed by a 10 min incubation period. The milk-ejection activity of this incubation medium was determined in a rat bioassay[13], in which lysine-vasopressin was approximately 4 times less potent than oxytocin; no attempt was made to differentiate between the two hormones, and results are expressed in international units (U) of oxytocin. The neurophysin content of the same incubation media was determined in triplicate by radioimmunoassay[7,8].

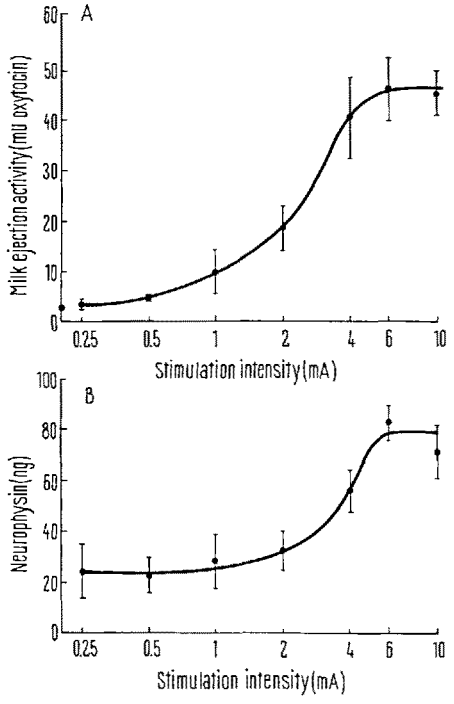

Fig. 1. Relation between stimulation intensity and output from isolated rat neurohyphyses. The points show hormone (A) and neurophysin (B) output in 10 min period of incubation following a 30 sec train of electrical stimuli (50 Hz, 2 msec) applied to the pituitary stalk at the indicated intensity. Points are mean ± S.E.M. of 4–6 responses obtained in separate experiments, each on a different neurohypophysis.

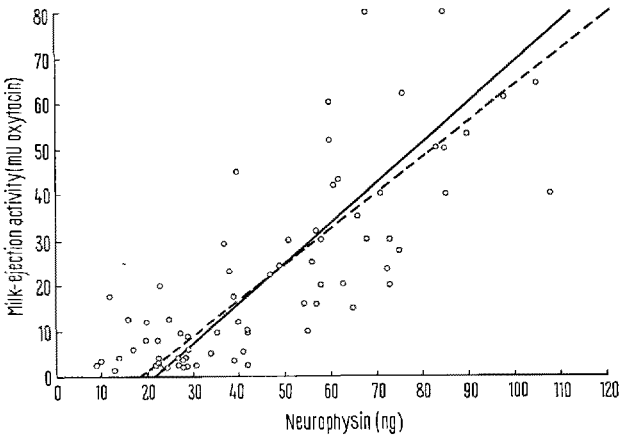

Fig. 2. Relation between hormone and neurophysin output following electrical stimulation of isolated neurohypophyses. Data are from 72 incubation periods in 54 single neurohypophyses. The neurophysin output (ng/gland/10 min) during each incubation is plotted along the abscissa, the hormone output (mU oxytocin/gland/10 min) along the ordinate. Regression lines have been drawn for $x = f(y)$, and $y = f(x)$.

[1] W. W. DOUGLAS and A. M. POISNER, J. Physiol., Lond. *172*, 1 (1964).
[2] M. GINSBURG and M. IRELAND, J. Endocr. *35*, 289 (1966).
[3] W. W. DOUGLAS, Br. J. Pharmac. *34*, 451 (1968).
[4] J. NAGASAWA, W. W. DOUGLAS and R. A. SCHULZ, Nature, Lond. *227*, 407 (1970).
[5] C. R. DEAN and D. B. HOPE, Biochem. J. *104*, 1082 (1967).
[6] C. R. DEAN, D. B. HOPE and T. KAŽIĆ, Br. J. Pharmac. *34*, 192P (1968).
[7] J. J. LEGROS, P. FRANCHIMONT and J. C. HENDRICK, C. r. Séanc. Soc. Biol., Paris *163*, 2773 (1969).
[8] J. J. LEGROS, in *Radioimmunoassay Methods* (Eds. K. E. KIRKHAM and W. M. HUNTER; Livingston, Edinburg 1971), p. 228.
[9] K. W. CHENG and H. G. FRIESEN, Endocrinology *88*, 608 (1971).
[10] K. W. CHENG and H. G. FRIESEN, Metabolism *19*, 876 (1970).
[11] J. J., DREIFUSS, J. D. GRAU and R. E. BIANCHI, *Experientia* 27, 1295 (1971).
[12] J. J. DREIFUSS, I. KALNINS, J. S. KELLY and K. B. RUF, J. Physiol., Lond. *215*, 805 (1971).
[13] G. W. BISSET, B. J. CLARK, J. HALDAR, M. C. HARRIS, G. P. LEWIS and M. ROCHA E SILVA, Br. J. Pharmac. *31*, 537 (1967).

Figure 1 shows that the milk-ejection activity and the neurophysin content of the incubation media following electrical stimulation are related to stimulation intensity, the 2 curves being S-shaped. In Figure 2, results from each individual incubation have been plotted according to its neurophysin (abscissa) and hormone (ordinate) content. The 2 regression lines ($x = -14.6 + 0.79y$, and: $y = 21.4 + 1.14x$) have been computed; their correlation coefficient is equal to 0.95. The fact that the intercept is different from zero is not uncommon when biological and immunoreactive potencies of substances are determined, and may also depend on the different sensitivities of the 2 assays.

Studies on the binding of the polypeptide hormones to neurophysins[3,5] have led to the suggestion that neurophysins are involved in the intracellular storage and transport of the hormones; their fate during the secretory process has however long remained obscure. The present study indicates that neurophysins and polypeptide hormones are released in a fixed ratio from isolated rat neurohypophyses over at least a 10-fold range of hormone output. UTTENTHAL et al.[14] have recently shown that isolated porcine neurohypophyses liberate lysine-vasopressin and neurophysin when exposed to solutions containing a high KCl concentration. These results suggest that the determination of plasma neurophysin levels could serve as a clinically useful index of the secretory activity of the neurohypophysis, since the assay of the very small quantities of polypeptide hormones in blood poses important technical problems. It remains to be shown however that the ratio is always fixed with different experimental conditions, and whether it reflects the ratio found in the neurosecretory granules[15].

Résumé. La stimulation électrique de neurohypophyses de rat in vitro provoque la libération conjointe d'hormones et de neurophysine. Cette observation suggère que la secrétion neurohormonale implique l'extrusion du contenu des granules neurosecrétoires.

J. J. NORDMANN, J. J. DREIFUSS and J. J. LEGROS[16]

Department of Physiology, University of Geneva Medical School, 20, rue de l'Ecole de Médecine, CH-1211 Genève 4 (Switzerland); and Radioimmunological Laboratory, Institute of Medicine, University of Liège, Liège (Belgium), 26 August 1971.

[14] L. O. UTTENTHAL, B. G. LIVETT and D. B. HOPE, Phil. Trans. R. Soc. B *261*, 379 (1971).

[15] This work was supported by grants from the Swiss National Science Foundation No. 5340.3 and from the F. Hoffmann-La Roche Foundation No. 117. The technical assistance of Miss R. E. BIANCHI is acknowledged.

[16] Fellow of the Belgian F.N.R.S.

Subcellular Distribution of ^{32}P Labelled Phospholipids: Stimulation by Thytropar and Long Acting Thyroid Stimulator

Thytropar (TSH) elicits numerous biochemical responses in its target tissue-thyroid, both in vivo and in vitro[1,2]. Increased incorporation of ^{32}Pi into the phospholipids of thyroid is one of the earliest effects of TSH[3-7]. Long acting thyroid stimulator (LATS) present in the serum of patients suffereing with Graves' disease has also been shown to mimic the action of TSH on thyroidal phospholipid metabolism[8,9].

Subcellular distribution of ^{32}P-labelled phospholipids in control and TSH-stimulated thyroids has been studied by KOGL and VAN DEENEN[10] and KERKOF and TATA[11]. In the present studies subcellular fractionation and localisation of the radioactivity in the TSH and LATS stimulated thyroids have been attempted with a view to analyze the distributory pattern of the ^{32}P-labelled phospholipids in these organelles.

Materials and methods. Thytropar (TSH) was a generous gift of Armour Pharmaceutical Company, Chicago. LATS was gift of MRC, London. $Na_2H^{32}PO_4$ was the product of Isotope Division, BARC, India.

Bovine thyroids from freshly slaughtered animals were sliced (100–150 mg bits) and 5 g of bovine thyroid slices (pooled slices from at least 6 thyroids) were incubated in 150 ml *Tris*-Cl buffer (pH 7.4; 50 m*M*) containing NaCl 131 m*M*; KCl 5 m*M*, $MgSO_4$ 1.2 m*M*; $CaCl_2$ 0.8 m*M* and $Na_2H\ ^{32}PO_4$ – 1 mCi for 1 h at 37 °C in a metabolic shaker under 100% oxygen. TSH and LATS were used at a concentration of 100 mU/ml and 1.5 MRC milli units/ml respectively. After the incubation period, the slices were washed several times with 0.1 *M* KH_2PO_4 and normal saline. Subcellular fractions were prepared by the differential centrifugation of the thyroidal homogenate in 4 volumes of 0.25 *M* sucrose. The 1000 *g* sediment (10 min centrifugation) was discarded. The 10,000 *g* sediment obtained after 20 min centrifugation was taken as the mitochondrial fraction. The microsomal fraction was the 105,000 *g* sediment (1 h centrifugation). Lipids were extracted from the homogenate, subcellular fraction and a portion of the 105,000 *g* supernatant with 20 volumes of $CHCl_3$: CH_3OH (2:1 v/v) mixture and purified according to the method of FOLCH et al.[12]. The total lipid radioactivity was measured using 15.0 ml of the scintillator fluid (PPO-5 g and dimethyl POPOP-0.3 g in IL Toluene) and counted in Nuclear Chicago Liquid Scintillation Counter. Individual phospholipids were resolved on TLC and the phospholipid zones

[1] N. FREINKEL, *The Thyroid Gland* (Eds. R. PITT-RIVERS and W. R. TROTTER, Butterworths, London 1964), vol. 1, p.131.

[2] I. PASTAN, A. Rev. Biochem *35*, 369 (1966).

[3] M. E. MORTON and J. R. SCHWARTZ, Science *117*, 103 (1953).

[4] N. FREINKEL, Endocrinology *61*, 448 (1957).

[5] P. VILLKI, *Advanced Thyroid Research* (Ed. R. PITT-RIVERS; Pergamon Press, Oxford 1961), p. 231.

[6] T. W. SCOTT, S. M. JAY and N. FREINKEL, Endocrinology *79*, 591 (1966).

[7] M. ALTMAN, H. OKA and J. B. FIELD, Biochim. biophys. Acta *116*, 586 (1966).

[8] T. W. SCOTT, B. F. GOOD and K. A. FERGUSON, Endocrinology *79*, 949 (1966).

[9] G. BURKE, J. clin. Endocr. Metab. *28*, 1816 (1968).

[10] F. KOGL and L. L. M. VAN DEENEN, Acta Endocr. *36*, 9 (1961).

[11] P. R. KERKOF and J. R. TATA, Biochem. J. *112*, 799 (1969).

[12] J. FOLCH, M. LEES and G. H. SLOANE-STANLEY, J. biol. Chem. *226*, 497 (1957).