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INTRA-AXONAL TRANSPORT AND TURNOVER OF NEUROHYPOPHYSIAL HORMONES IN THE RAT

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SUMMARY

1. The specific radioactivities of isotopically pure oxytocin and vasopressin prepared from the neural lobe of the pituitary gland have been measured at various times after an intracisternal injection of [^{3}H]tyrosine.

2. Radioactive hormone began to appear in the gland 1–2 hr after the injection which suggests an intra-axonal transport velocity of 1–2 mm/hr.

3. From 7 days onwards the specific radioactivity of each hormone declined exponentially with the same rate constant and a half-life of about 13 days.

4. If the decline in radioactivity can be equated with the release of the hormones, the rates of secretion for the male rat in water balance are 18·7 m-u./day for oxytocin and 28·9 m-u./day for vasopressin.

5. Calculation from the secretion rates gave steady-state plasma concentrations of about 3 μu./ml. for each hormone.

INTRODUCTION

In the hypothalamo-neurohypophysial neurosecretory system, the hormones oxytocin and vasopressin are synthesized in the hypothalamus and then transported along the hypothalamo-neurohypophysial tract for storage in the neural lobe of the pituitary gland (Scharrer & Scharrer, 1954). Sachs and his colleagues have shown (for review see Sachs, 1969; Sachs, Fawcett, Takabatake & Portanova, 1969) that the hypothalamus is capable of synthesizing vasopressin, but until recently measurements of intra-axonal transport rates have relied on histo-autoradiography. After injection of radioactive cysteine into the subarachnoid space, radioactive material was seen to pass along the fibres of the tract and to reach the neural lobe some 5–10 hr later (Sloper, Arnott & King, 1960; Flament-Durand, 1961, 1967).

The amounts of hormone stored in the gland are relatively large when compared with the estimated rate of daily release under normal physio-

logical conditions (Lederis & Jayasena, 1970). For example, the gland contains sufficient vasopressin to last the animal for several weeks, and so the turnover rate of glandular hormone might be expected to be rather low. Indeed, the observation that radioactive vasopressin could still be found in the neural lobe of the dog 12 days after an intraventricular injection of [^{35}S]cysteine (Sachs & Haller, 1968) is consistent with a slow turnover.

We have described a method for the isolation of isotopically pure oxytocin and vasopressin from small groups of rats after intracisternal injection of [^{3}H]tyrosine (Pickering & Jones, 1971*a*). The present paper describes attempts to use this method to estimate intra-axonal transport rates and to measure turnover, by following the build up and decline of radioactive hormone after the injection of radioactive amino acid into the cerebrospinal fluid. Preliminary reports of some of these results have been given already (Jones & Pickering, 1970; Pickering & Jones, 1971*b*).

METHODS

Animals. Male rats (180–220 g) were used from the departmental colony of Wistar animals of the Porton Strain (derived M.R.C. Carshalton).

Radioactive tyrosine. [^{3}H]tyrosine of high specific activity (L-tyrosine-3, 5-T, TRK 200) was obtained from the Radiochemical Centre, Amersham. The batches used in the present experiments had specific activities between 36 and 43 Ci/m-mole.

Intracisternal injection. Rats were anaesthetized with ether and fixed in the head-piece of a stereotaxic instrument. A needle, attached to a syringe containing [^{3}H] tyrosine solution, was inserted into the *cisterna magna* and 0·1 ml. of the radioactive solution (0·1 mCi) was injected over a period of 8 min. The animal was then allowed to recover from the anaesthetic; the total duration of anaesthesia was about 15 min.

Removal of hypothalamic blocks. A block of hypothalamic tissue containing the supraoptic and paraventricular nuclei was removed essentially as described by Bie & Thorn (1967). Using a fine pair of scissors cuts were made: anteriorly, immediately rostral to the optic chiasma; posteriorly, immediately anterior to the pons; laterally, at the lateral edges of the cerebral peduncles. The cuts were deepened vertically into the brain and a parallelepiped of tissue removed. The dorsal aspect of this block was then trimmed down to just above the roof of the third ventricle.

Preparation of hypothalamic extracts. The blocks of hypothalamic tissue were homogenized in 10 ml. 0·05 M acetic acid. A portion (0·1 ml.) was removed for the determination of radioactivity and proteins were precipitated from the remainder by the addition of 2 ml. 60 % (w/v) trichloroacetic acid (TCA). After allowing to stand overnight at 4 °C the protein-free extract was recovered by centrifuging at 3000 *g* for 30 min.

Isolation of isotopically pure hormones. Neural lobes were pooled from four or five animals and extracted with acetic acid (0·25 % w/v). Isotopically pure oxytocin and vasopressin were then recovered from the extracts by a combination of ion-exchange chromatography on Amberlite CG-50 and on carboxymethylcellulose. These methods have been described in detail already (Pickering & Jones, 1971*a*).

Determination of specific radioactivity. Radioactivity was determined with a

Packard Tri-Carb Scintillation Spectrometer as described previously (Pickering & Jones, 1971*a*) and converted to disintegrations per minute (d.p.m.) by external standardization. Biological activity was estimated as rat vasopressor activity (Dekanski, 1952) and rat uterus activity (Holton, 1948; Munsick, 1960) respectively. Specific radioactivity was then expressed as d.p.m./m-u.

RESULTS

Changes in radioactivity in the hypothalamus

In order to gain an approximate indication of the degree of labelling of the hormone synthesis sites, blocks of hypothalamic tissue were homogenized and their radioactivity measured before and after precipitation of proteins with TCA. The results are shown in Fig. 1 and are expressed

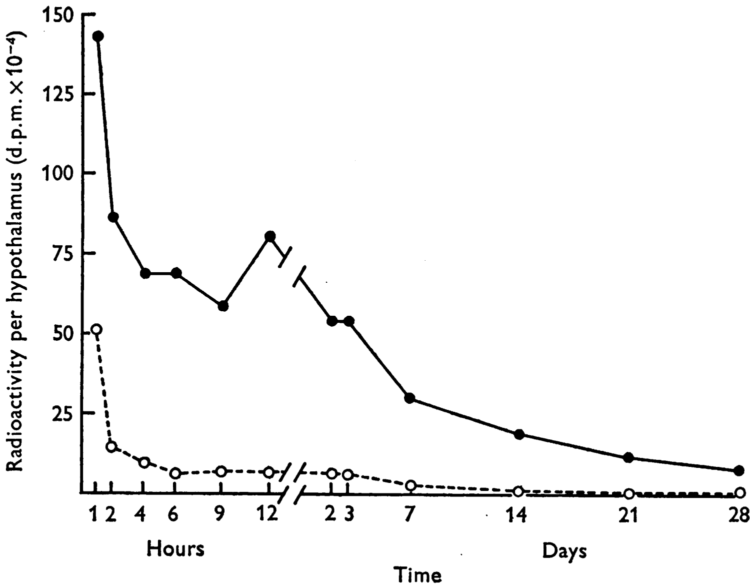

Fig. 1. Disappearance of radioactivity from the hypothalamus at various times after an intracisternal injection of 100 μCi [^{3}H]tyrosine. Hypothalamic homogenates were prepared and protein precipitated with trichloroacetic acid as described in Methods. ●—●, radioactivity in total homogenate. ○—○, radioactivity remaining in supernatant after precipitation.

in terms of radioactivity per hypothalamic block. The blocks were cut (see Methods) to include the whole of the paraventricular and supraoptic nuclei. One hour after the injection of labelled amino acid, 64 % of the hypothalamic radioactivity was already precipitable by trichloroacetic acid. Thus the level of TCA-soluble radioactivity, which would include free [^{3}H]tyrosine, fell dramatically within the first few hours of the experiment.

Changes in the labelling of neurohypophysial oxytocin and vasopressin

Table 1 shows the specific radioactivity of oxytocin and vasopressin isolated from rat neural lobes at various times after the animals had received intracisternal injections of [^{3}H]tyrosine. The specific radioactivity of each hormone began to rise between 1 and 2 hr after the injection and reached a maximum value at 6–12 hr.

TABLE 1. Specific radioactivity of isotopically pure oxytocin and vasopressin isolated from the neural lobes of rats at various times after an intracisternal injection of 100 μCi [^{3}H]tyrosine

Time after injection (hr)	Specific radioactivity (d.p.m./m-u.)	
	Oxytocin	Vasopressin
1	0·028; 0·052; 0·10; 0·23	0·075; 0·021; 0·048; 0·039
2	0·53; 0·86; 1·21; 1·79	1·38; 1·08; 1·59; 0·72
4	2·25; 2·63; 2·63; 2·05	3·17; 4·87; 2·55; 4·12
6	2·66; 5·51; 1·97; 1·41; 2·16	4·54; 8·24; 6·14; 3·63; 3·69
9	1·96; 1·71; 1·73	4·61; 3·65; 4·43
12	3·27; 4·45; 4·78; 1·59	4·57; 11·06; 8·24; 3·75
48	1·85; 1·43; 3·08	3·55; 4·31; 4·58
72	1·96; 2·29; 1·58	3·59; 3·77; 4·99
168	0·82; 4·45; 1·05; 2·17	3·33; 3·31; 2·29; 1·65
336	0·49; 1·13; 0·77; 0·59	1·21; 3·60; 1·06; 1·42
504	0·30; 1·01; 0·58	0·81; 1·32; 1·22
672	0·70; 0·54	0·99; 0·66

From 12 hr the specific radioactivities fell slowly throughout the following 4 weeks. The decline in specific radioactivity was analysed from 7 days onwards, by which time possible effects of the injection would have worn off, and found to be exponential as shown by the linear decline of the logarithms with time (Fig. 2). The regression lines shown in Fig. 1 were fitted by the least squares method and are described by the equations

$$\log_{10} S_{\mathrm{oxy}} = 0{\cdot}2960 - 0{\cdot}0232\, t, \tag{1}$$

$$\log_{10} S_{\mathrm{vpr}} = 0{\cdot}5596 - 0{\cdot}0242\, t, \tag{2}$$

where S_{oxy} and S_{vpr} are the specific radioactivities of oxytocin and vasopressin respectively, and t is time after injection, in days.

These correlations were significant for both oxytocin ($P < 0{\cdot}05$) and vasopressin ($P < 0{\cdot}01$) and the slopes of the lines were not significantly different. Thus from 7 days after the injections onwards, radioactive vasopressin and oxytocin disappeared from the gland at about the same rate with a half-time of approximately 13 days ($t\frac{1}{2}$, $_{\mathrm{oxy}} = 13{\cdot}0$; $t\frac{1}{2}$, $_{\mathrm{vpr}} = 12{\cdot}5$). Since the hormone content of the neurohypophysis remains essen-

tially constant (Heller, 1966), the disappearance of hormone from the gland must be balanced by the arrival of freshly synthesized hormone. In the present experiments about 90 % of the radioactivity present in the hypothalamus after 6 hr was TCA-precipitable, so that at later times radioactive hormone disappearing from the neural lobe is probably being

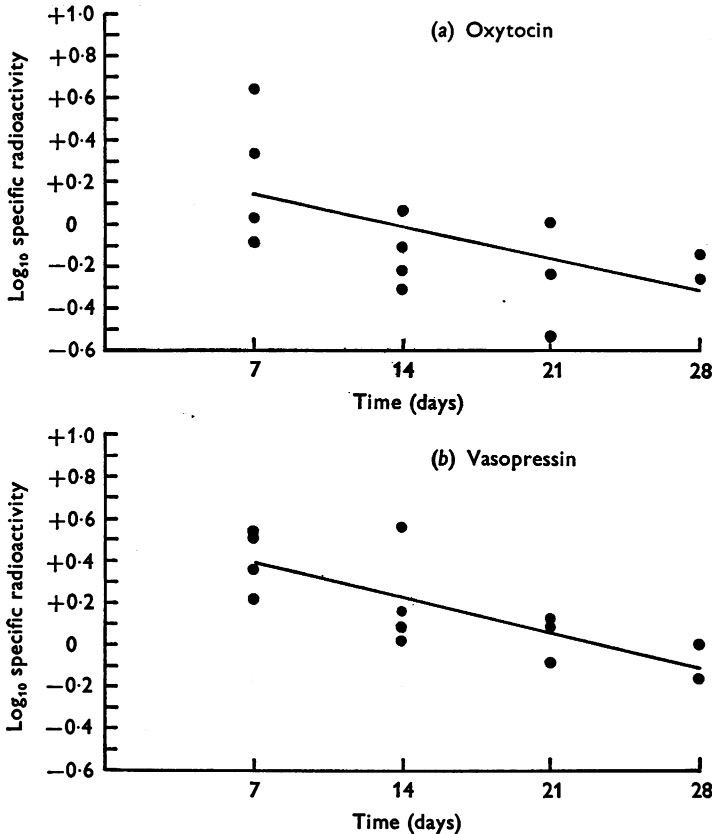

Fig. 2. Disappearance of (*a*) radioactive oxytocin and (*b*) radioactive vasopressin from the neural lobe of the rat. The logarithm of the specific activity of each hormone is plotted against the time after an intracisternal injection of 100 μCi [^{3}H]tyrosine. The lines of best fit have been drawn for each hormone and were calculated by the least-squares method.

replaced by newly synthesized unlabelled hormone from the hypothalamus. The change in specific radioactivity of the hormone in the gland can therefore be expressed as

$$\frac{dS}{dt} = \frac{-R}{H}S, \tag{3}$$

where S = specific radioactivity of the hormone, H = hormone content of the gland, R = amount of hormone disappearing in time t, or

$$\log_{10} S = \text{constant} - \frac{R}{2{\cdot}303H}t. \quad (4)$$

Eq. (4) describes the exponential fall of specific activity with time, and is thus another form of the functions plotted in Fig. 2. We can therefore use eqs. (1), (2) and (4) to solve for R, so that

$$R_{\text{oxy}} = 2{\cdot}303\, H_{\text{oxy}} \times 0{\cdot}0232 \text{ m-u./day}$$

and

$$R_{\text{vpr}} = 2{\cdot}303\, H_{\text{vpr}} \times 0{\cdot}0242 \text{ m-u./day}.$$

The gland contents (H) of an average 200 g rat of our strain may be assumed to be 350 m-u. oxytocin and 520 m-u. vasopressin (Jones & Pickering, 1969) and thus the rates of disappearance (R) of oxytocin and vasopressin are 18·7 m-u./day and 28·9 m-u./day respectively, i.e. about 5 % gland content/day.

DISCUSSION

Radioactive hormone began to arrive in the neural lobe of the pituitary gland between 1 and 2 hr after the intracisternal injection of the labelled amino acid (Table 1). Sloper (1958) has estimated the length of the hypothalamo-neurohypophysial tract in the rat to be about 2 mm, so that our results would suggest a transport rate of 1–2 mm/hr. This may not represent the true resting rate of transport, however, since synthesis and transport may have been stimulated during anaesthesia and intracisternal injection. On the other hand, this transport rate has been calculated from the total time for synthesis and transport, and Takabatake & Sachs (1964) showed that during the biosynthesis of vasopressin by the hypothalamus there was a lag-period of 1–1½ hr before newly synthesized hormone became detectable. They suggested that this interval represented the time for synthesis and break-down of a protein precursor. If these processes are completed before the newly synthesised hormone leaves the perikaryon, the present result would suggest a much faster transport rate (>4 mm/hr). If, however, the maturation of precursor occurs *en route* from the site of synthesis in the perikaryon to the release site in the nerve terminal (Sachs *et al.* 1969; Pickering, Jones & Burford, 1971) then the true transport rate would be somewhere between these two extremes. Whatever the true value for the transport rate, it is evident that the neurohypophysial hormones fall into the group of neuronal products which is rapidly transported along axons (see Barondes, 1967) rather than that which is carried passively by the axoplasmic flow described by Weiss and his colleagues (Weiss, 1944, 1968; Weiss & Hiscoe, 1948).

As well as the hormones the neurosecretory granules contain the so-called carrier proteins – the neurophysins (see Ginsburg, 1968) – and there is mounting evidence that these proteins are released along with the hormones (Uttenthal, Livett & Hope, 1971; McNeilly, Legros & Forsling, 1972). Norström & Sjöstrand (1971*a*) found that a rat neurohypophysial protein which cross-reacted with antibodies raised against porcine neurophysin (Norström, Sjöstrand, Livett, Uttenthal & Hope, 1971) also first became labelled about 2 hr after injection of [^{35}S]cysteine into the brain in the region of the supraoptic nucleus. Calculations from the results of Norström & Sjöstrand (1971*b*) show that the radioactivity of this protein declined with a half-life of about 19 days when measured between 7·5 and 9 days after injection of the isotope. Within the experimental error the decline of specific radioactivities of the hormones agrees well with this value. We have been able to resolve two major neurophysins from rat neurohypophysial extracts (Burford, Jones & Pickering, 1971), and after intracisternal injections of [^{35}S]cysteine the pattern of labelling for both of the proteins is very similar to those shown in Table 1 for the hormones (G. D. Burford & B. T. Pickering, unpublished observations).

These more biochemical approaches have suggested rather higher rates of intra-axonal transport than those derived from the original autoradiographic studies (Sloper *et al.* 1960; Flament-Durand, 1961, 1967). This discrepancy probably reflects differences in the sensitivities of the methods and, indeed, more recent autoradiographic studies with the electron microscope indicated a transport rate which is more compatible with the faster estimate (Nishioka, Zambrano & Bern, 1970).

The secretion rates which we have calculated from the rates of disappearance of radioactive hormones from the gland (19 m-u./day for oxytocin and 29 m-u./day for vasopressin, or about 5 % gland content) are not very different from those estimated by others from extrapolation of estimated daily requirements (see Lederis & Jayasena, 1970). In making these calculations we have assumed that all of the hormone which disappears from the gland is secreted. Thus it would appear that at least under non-stimulated conditions (i.e. in the male rat in water balance), only a very small proportion of the neurohypophysial hormone store is removed by lysosomes acting as a mechanism for the control of excess hormones as suggested by Smith & Farquar (1966) for the anterior pituitary. Lysosomal activity in the neural lobe increases rather than decreases under conditions calling for increased release of hormone (Kobayashi, Oota & Hirano 1962; Whitaker & LaBella, 1972) in keeping with the suggestion that the function of lysosomes in the nerve terminals of the neural lobe is to metabolize the recaptured granular membranes after the release of granule contents rather than to destroy excess hormone (Whitaker & LaBella, 1972).

If the disappearance of hormone from the gland is equated with a steady release into the blood stream, then the plasma concentration of each hormone can be calculated using the volume of distribution and the half-time for disappearance from the blood.

The total amount of circulating hormone, which may be assumed to remain constant under resting conditions, can be expressed as CV μu. (where C = plasma concentration, and V = volume of distribution of the hormone). The rate of disappearance of hormone from the circulation may then be expressed as kCV μu./min, where k is the rate constant for clearance, and is related to the half-life ($t_{\frac{1}{2}}$) of the hormone in plasma by the expression

$$k = \frac{2{\cdot}303\ \log_{10}S}{t_{\frac{1}{2}}}\ \text{min}^{-1}. \tag{5}$$

Thus the rate of disappearance of hormone from the constant circulating pool can be expressed as

$$\frac{0{\cdot}693\,CV}{t_{\frac{1}{2}}}\ \mu\text{u./min}.$$

To maintain the pool at a constant level this rate of loss must be balanced by the rate of release (r) of hormone from the gland so that

$$r = \frac{0{\cdot}693\,CV}{t_{\frac{1}{2}}}$$

or

$$C = \frac{rt_{\frac{1}{2}}}{0{\cdot}693\,V}\ \mu\text{u./ml}. \tag{6}$$

Thus by converting the rate of disappearance (R) of hormone from the gland calculated from eq. (4) into μu./min (r), eq. (6) can be used to obtain an estimate of the plasma concentration of each hormone.

The volume of distribution of the hormones may be taken at 7·3 ml./100 g rat and the half-life of oxytocin as 2 min (Fabian, Forsling, Jones & Lee, 1969) in which case the observed rate of disappearance of oxytocin from the gland would result in a plasma oxytocin concentration (in the 200 g rat) of 2·6 μu./ml. Selecting a half-life for arginine vasopressin is more difficult since a number of values ranging from 0·7 to 3·4 min have been published (see Ginsburg, 1968). Taking an approximate average of this range as 2 min, which in fact would be the half-time estimated by Czackes & Kleeman (1964) at the blood concentration in resting rats, the plasma concentration calculated from the observed disappearance rate of vasopressin comes to 4·0 μu./ml. Neglecting the findings of Czackes & Kleeman (1964), however, the half-time for vasopressin would be closer to 1 min (see Ginsburg 1968) so that the plasma concentration of the hormone would be nearer 2·0 μu./ml. These values have been calculated from results

obtained at least 7 days after anaesthesia and injection, and so may be regarded as the basal levels for the male rat in water balance.

The estimation of plasma concentrations of neurohypophysial hormones in the resting animal by direct assay is complicated by the fact that the secretion of the hormones is extremely sensitive to anaesthetic and to surgical intervention (Heller & Ginsburg, 1966). Moreover, until recently, the resting levels have been below the limits of sensitivity of available assay methods. However, normal blood levels of the order of 10 μu./ml. have been postulated (see Fitzpatrick & Bentley, 1968) and, using sensitive methods, so-called resting levels of vasopressin in plasma have been estimated as 1·5–4 μu./ml. in the rat (Tata & Buzalkov, 1966), 0·5–2 μu./ml. in man (Forsling, Jones & Lee, 1968) and 5 μu./ml. in the goat (McNeilly *et al.* 1972). Thus the plasma concentrations calculated in the present experiments, which may be considered to be relatively unaffected by surgery, are compatible with those found by direct assay. This agreement provides further evidence that the calculated release-rates of the hormones are not very different from the true release-rates.

The variation of the experimentally derived specific activities does not allow us to draw any precise conclusions about the number of hormone pools within the gland. There is considerable evidence that the hormones are not stored in a single homogeneous pool in the neural lobe, but that there is a small pool of 'readily releasable' hormone (Sachs, Share, Osinchak & Carpi, 1967). Moreover, it seems likely that newly synthesized hormone may enter this pool first (Sachs & Haller, 1968). Our results suggest that the 'readily releasable' pool, which has been demonstrated during release due to acute stimuli, may not play a major part in the loss of hormone from the gland under resting conditions. Even allowing for the amount of variation, there is no indication of an initial fast rate of decline of specific radioactivity followed by a later slower one.

Norström & Sjöstrand (1971*b*) studied the turnover of a neurohypophysial protein related to neurophysin and have interpreted their results as showing a slow and a fast component of intra-axonal transport for neurosecretory products. They obtained two peaks of radioactivity in their protein from the neural lobe after injection of radioactive amino acid into the hypothalamus: one at about 24 hr and the other at about 3 days after injection. Our results provide no clear-cut support for a two rate transport system of this kind.

The present study has given an overall picture of the turnover of neurohypophysial hormones, but it is a very time-consuming approach and is perhaps not precise enough for detailed mathematical analysis. The two major neurophysins of the rat can be readily separated by polyacrylamide gel electrophoresis (Burford *et al.* 1971; Burford & Pickering,

1972). One of these appears to be biosynthetically linked with oxytocin and the other with vasopressin. We are currently determining the rates of transport and turnover of each of these individual proteins and it will be interesting to see if they parallel those of the hormones. The radioactivity of the proteins can be determined more precisely than that of the hormones, so that these results should be more amenable to mathematical analysis.

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