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RELEASE OF VASOPRESSIN AND OXYTOCIN FROM ISOLATED PITUITARY GLANDS OF ADULT AND NEW-BORN RATS

BY S. E. DICKER

From the Department of Physiology and Pharmacology, Chelsea College of Science, London, S.W. 3

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SUMMARY

1. Pituitary glands of adult rats of both sexes, of lactating female and of new-born rats, incubated in a Locke solution, release both oxytocin and vasopressin. The amount of hormones released, during a measured period of incubation, is related to the actual hormone content of the gland.

2. Increasing the concentration of KCl in the incubation medium, with $CaCl_2$ present and in concentration of at least 2·2 mM, produces an enhanced release of both hormones from pituitary glands of adults, but does not affect the release of hormones from glands of new-born animals.

3. Addition of ouabain to the incubation medium produces a marked increase of the release of the hormones from glands of both adult and new-born rats. This is accompanied by an extrusion of K ion and an influx of Na ion. The effect of ouabain on the hormone release and the shift of ions can be reversed by subsequent addition of adenosine triphosphate.

4. The increased release of hormones produced by ouabain, in glands from new-born rats, is unaffected by the presence or absence of $CaCl_2$. In adults, however, the effect of ouabain, though present, is reduced in the absence of $CaCl_2$.

5. It is suggested that in glands from adult animals, the hormones must be freed from their attachment on the protein-carrier, neurophysin and that this can be achieved by the entry of calcium ion into the cell. The subsequent secretion of the 'freed' hormones appears to be accompanied by a shift of ions across the cell membrane.

6. In glands from neonates up to 3 weeks old, the absence of neurophysin, or its poor capacity for binding the hormones, explains the inability of calcium to operate in the same way as in the glands of adults. There is evidence suggesting that the secretion of the neurohypophysial hormones in the new-born animal consists mainly of their diffusion from the cells, without previous elution of the hormones as in adults.

INTRODUCTION

The neural lobe of the pituitary gland of mammals is known to be the site of storage and release of the hormones, vasopressin and oxytocin. Histologically, the neural lobe has been described as an aggregate of secretory terminals of cells located in the supraoptic and paraventricular nuclei of the hypothalamus (Scharrer, 1954). Factors which in the animal evoke the release of the neurohypophysial hormones act through the neurosecretory cells of the hypothalamus; hence the suggestion that the release occurs as a result of impulses discharged along the hypothalamo-hypophysial tract.

According to recent evidence, both hormones are adsorbed on an inert protein, neurophysin, with which they appear to travel along the axons and with which they are stored ultimately in the posterior lobe. If this is so one would expect that the actual process of secretion must be preceded by that of 'elution', unless one accepts the view that both hormones and carrier are released together into the blood stream (Scharrer, 1954).

The present investigation was already in progress when Douglas (1963) and Douglas & Poisner (1963, 1964*a*) published the results of their experiments showing that the secretion of vasopressin involves the depolarization of the neurosecretory terminals followed by an increased uptake of calcium. The authors, however, limited their investigation to the release of vasopressin. As it is known that in the great majority of cases the stimulation of the neurohypophysial complex leads to the simultaneous secretion of both vasopressin and oxytocin, it was thought of interest to see whether the same mechanism applied to the secretion of both hormones and to extend the investigation from adult rats of both sexes to lactating female and new-born rats.

METHODS

Male and female adult white Wistar rats, female lactating and newly born rats were used. The female lactating rats were killed after 18 days of lactation, at a time when the highest vasopressin/oxytocin ratio was expected to be found in their neural lobe (Dicker & Tyler, 1953*a*, *b*; Acher & Fromageot, 1956). The new-born animals varied in age from 1 to 21 days. Adult rats were killed by dislocation of the neck, neonates by decapitation.

As soon as the animals were killed the pituitary glands were dissected out, removed and immersed in a phosphate-buffered Locke solution, pH 7 (NaCl, 154 mM; $CaCl_2$, 2·2 mM; KCl, 5·6 mM; NaH_2PO_4, 2·15 mM; glucose: 10 mM). For adult animals, five glands were pooled, for neonates twenty. The volume of solution in which the glands were incubated was 1·0 ml., the temperature 37° C. During the incubation the glands were shaken continuously, gassed with O_2 or with air. After 20 min of incubation the immersion fluid was removed and replaced by 1·0 ml. of fresh solution. This was repeated every 20 min. The first two samples were discarded; after this, solutions were assayed for both their oxytocin and vasopressin content and the result expressed as m-u./20 min/5 or 20 glands, as the case might be. In preliminary experiments, the posterior lobe of the hypophysial gland was dissected from the anterior lobe, the former being used only. Later the whole gland was used. In contrast with

Douglas & Poisner's (1964*a*) technique, the glands were not bisected, but were incubated whole: this may account for some quantitative differences between Douglas & Poisner's (1964*a*) estimations and those presented here.

The oxytocic activity was assayed on an isolated rat uterus perparation, according to Holton's technique (1948); the pressor activity was estimated using the blood pressure of an anaesthetized rat, as described by Dekanski (1952). They were assayed against standard solutions of synthetic oxytocin (Syntocinon, Sandoz Ltd.) and of a commercial preparation of vasopressin (Pitressin, Parke Davis and Co.). The presence of either oxytocic or pressor activity in the incubation medium were taken as indication of release of the hormones.

For the estimation of Na and K content of the tissues, glands were digested in 0·2 ml. of 16 N-HNO_3, overnight. When the glands were fully digested, the solution was made up to a volume of 15 ml. with distilled water. Na and K were estimated by flame photometer and expressed as m-equiv./l. Two controls, one of HNO_3 diluted with water and one of water only, were used regularly.

RESULTS

Since it was not technically possible to separate the posterior from the anterior lobe in hypophysial glands of new-born rats, comparison between data obtained from both adult and new-born animals was made by using whole undivided glands only. In order to establish the normal rate of release of both hormones, the glands were incubated for periods of 20 min to 2 hr. The first two periods of incubation were discarded (see Methods) and the hormone activities were assayed in the succeeding samples. In seventy-eight experiments, using pituitary glands of male and female adult rats (range of body weight: 220–280 g), the amount of vasopressin released was $12{\cdot}3 \pm 0{\cdot}77$ m-u./20 min/5 glands and that of oxytocin $28{\cdot}7 \pm 1{\cdot}81$ m-u./20 min/5 glands.

Effects of changes in the osmotic pressure. An increase of the osmotic pressure of the blood causes a secretion of both oxytocin and vasopressin, *in vivo*. In order to see whether a change of the osmotic pressure of the incubation medium had an effect on the release of the hormones, the NaCl content of the standard solution was either raised from 154 to 308 mM (five experiments) or decreased to 108 mM (four experiments). Neither the increase nor the decrease of osmotic pressure had an effect on the release of the hormones.

Effects of acetylcholine. In the intact animal the release of the hormones from the neurosecretory terminals is mediated by acetylcholine. This was tested on the isolated glands, *in vitro* by adding acetylcholine (10^{-4} g/ml.) to the incubation solution in the presence of eserine (10^{-5} g/ml.) (five experiments), and in the absence of the latter (three experiments). The addition to the incubation medium of acetylcholine had no effect on the release of vasopressin or oxytocin, confirming the observations of Douglas & Poisner (1964*a*) in respect of vasopressin.

Effects of excess potassium. According to Douglas & Poisner (1964*a*) an increase of the K concentration in the incubation medium from 5·6 to

56 mM produces a 10-fold increase in the amount of vasopressin released. In the following experiments concentrations of KCl 5·6, 28, 56 and 112 mM were used. The concentration of all other ions remained unchanged. A fivefold increase of KCl concentration (28 mM) had no significant effect on the release of either vasopressin or oxytocin (five experiments). When the concentration of KCl was raised further to 56 (ten experiments) and 112 mM (ten experiments), the amounts of oxytocin released increased from 28·7 to $59 \pm 2{\cdot}4$ and $75{\cdot}4 \pm 1{\cdot}25$ m-u./20 min/5 glands and those of vasopressin from 12·3 to $27{\cdot}6 \pm 0{\cdot}98$ and $39{\cdot}6 \pm 0{\cdot}92$ m-u./20 min/5 glands, respectively.

Effects of calcium on the release of the hormones. Douglas & Poisner (1964*a*) had shown that the enhanced release of vasopressin observed when posterior pituitary glands were incubated in a medium containing an excess of potassium ion occurred only in the presence of calcium. It was thought of interest to see whether changes in calcium ion alone had similar effects. Glands were incubated in the usual manner, in a calcium-free 'Locke' solution and in solutions containing 0·55, 1·1, 2·2, 4·4 and 8·8 mM-$CaCl_2$, the concentrations of other ions remaining unchanged. In the absence of calcium ion, or with concentrations of $CaCl_2$ below 2·2 mM, the rate of oxytocin output remained below that observed in control experiments. In five experiments without Ca, and in ten experiments in which concentrations of $CaCl_2$ of 0·55 and 1·1 mM were used, the mean release of oxytocin was $13{\cdot}3 \pm 1{\cdot}02$, $17{\cdot}9 \pm 1{\cdot}81$ and $15{\cdot}6 \pm 1{\cdot}76$ mu./20 min./5 glands compared with $28{\cdot}7 \pm 0{\cdot}77$ in control periods. The release of vasopressin, however, did not appear to be affected; in calcium-free 'Locke' solution, it was $10{\cdot}1 \pm 0{\cdot}20$ (5) m-u./20 min/5 glands and with 0·55 and 1·1 mM-$CaCl_2$ present, $10{\cdot}9 \pm 0{\cdot}17$ (3) and $10{\cdot}3 \pm 1{\cdot}05$ (3) m-u./20 min/5 glands respectively. When concentrations of $CaCl_2$ were raised to 2·2, 4·4 and 8·8 mM, there was a progressive increase in the amounts of both oxytocin and vasopressin released (Fig. 1).

These observations led to the investigation of the effects of calcium ion in the presence of a high KCl concentration (56 mM). In all experiments in which the concentrations of $CaCl_2$ were lower than 2·2 mM, the increased K concentration had little effect on the release of the hormones. An enhanced release of the hormones occurred with concentrations of $CaCl_2$ of 2·2 mM; this was further accentuated with concentrations of 4·4 mM-$CaCl_2$. Doubling the concentration of $CaCl_2$ to 8·8 mM, however, reduced the amount of hormones released, a fact that Dougals & Poisner (1964*a*) had already observed for vasopressin (Fig. 2).

Effects of substituting barium ion for calcium ion. Addition of barium to the Locke solution used during the perfusion of acutely denervated cats' adrenal glands increases markedly the release of catecholamines (Douglas

& Rubin, 1964). In order to see whether barium would have a similar effect on the release of hormones from isolated pituitary glands, the $CaCl_2$ of the incubation solution was replaced by either 2·5 μM-$BaCl_2$ (seven experiments) or 5·0 μM-$BaCl_2$ (eight experiments). These concentrations were one thousand times smaller than those used by Douglas & Rubin (1964) on the adrenal glands. It was not possible to exceed these doses

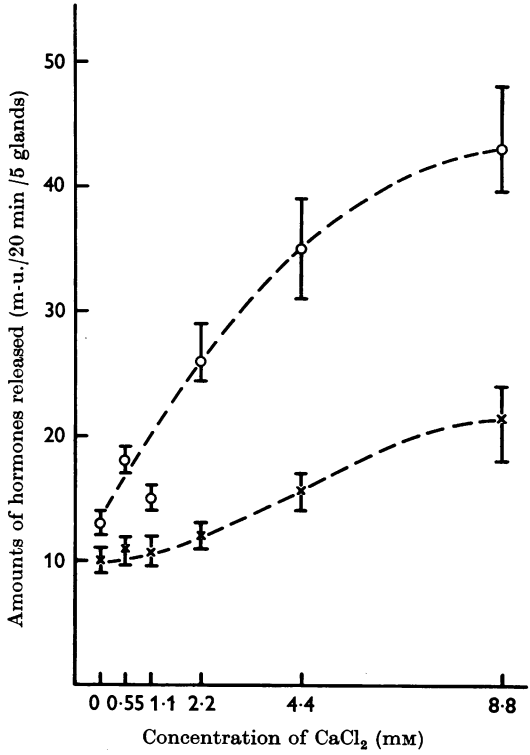

Fig. 1. Effect of increasing concentration of calcium ion on the release of vasopressin and oxytocin. Five pituitary glands were incubated for periods of 20 min, in a 'Locke' solution containing $CaCl_2$ at different concentrations. ○, oxytocin; ×, vasopressin. Vertical lines: ± S.E. of mean.

since increases in the concentration of $BaCl_2$ in the solution interfered with the quantitative assays of the hormones. The technique of the experiment was similar to that already described: glands incubated for two periods of 20 min in a standard Locke solution were transferred into a solution in which $CaCl_2$ had been replaced by $BaCl_2$ and incubated for another two periods of 20 min each. Oxytocin and vasopressin were estimated in the control samples and their amounts compared with those found in the $BaCl_2$ samples. The standard solutions of oxytocin and vasopressin were made up with the same concentration of $BaCl_2$ as control.

In spite of the low concentrations used, addition of $BaCl_2$ invariably produced a decrease in the output of oxytocin, but did not affect that of vasopressin. The release of oxytocin fell from a mean $31{\cdot}0 \pm 1{\cdot}97$ m-u./20 min/5 glands (12) in the control periods to $20{\cdot}6 \pm 2{\cdot}23$ m-u. htiw 2·5 μM-$BaCl_2$ and to $15{\cdot}0 \pm 0{\cdot}89$ m-u. with 5 μM-$BaCl_2$ (12). This inhibition of the release of oxytocin was accompanied by a fall in the Na and a rise of K content of the glands.

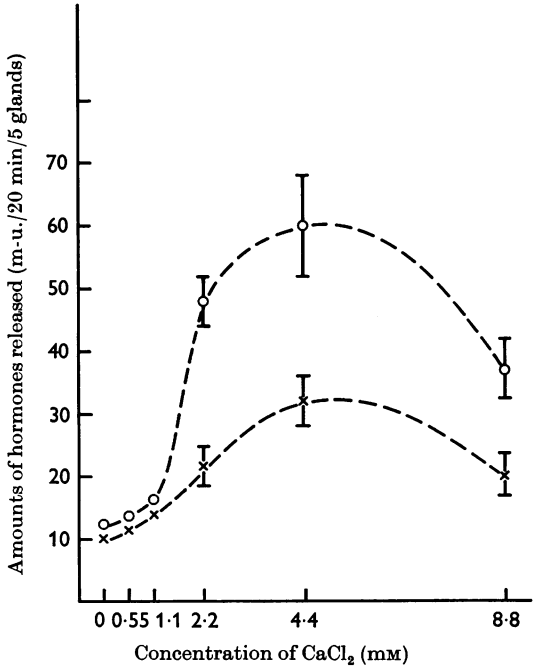

Fig. 2. Effect of increasing concentration of calcium ion in the presence of an excess potassium ion on the release of vasopressin and oxytocin. The incubation medium contained 56 mM-KCl the concentrations of calcium varied from 0 to 8·8 mM-$CaCl_2$. ○, oxytocin; ×, vasopressin; vertical lines: ±S.E. of mean. Note that below a concentration of $CaCl_2$ of 2·2 mM, a 10-fold increase of KCl had little effect on the release of the hormones (compare with Fig. 1).

In an attempt to investigate further the mechanism of action of barium ion, the concentration of KCl in the incubation solution was increased 10-fold to 56 mM, while that of $BaCl_2$ remained at 2·5 μM (six experiments). This produced a marked increase in the release of oxytocin which rose from $14{\cdot}9 \pm 1{\cdot}02$ m-u. during the control periods to $136{\cdot}1 \pm 2{\cdot}66$ m-u./20 min/5 glands. The release of vasopressin increased from $9{\cdot}6 \pm 1{\cdot}01$ m-u. to $53{\cdot}2 \pm 3{\cdot}15$ m-u/20 min/5 glands. Thus in the presence of excess KCl (56 mM), barium ion had a similar effect to that of calcium.

Effects of ouabain and ATP. Ouabain was used in the concentration of

0·5 mM. Its addition to the incubation medium produced a marked increase in the release of both hormones. The release of oxytocin rose from $29{\cdot}6 \pm 0{\cdot}52$ to $134{\cdot}9 \pm 5{\cdot}66$ (10) and that of vasopressin from $11{\cdot}0 \pm 0{\cdot}72$ to $57{\cdot}6 \pm 6{\cdot}20$ (10) m-u./20 min/5 glands. This was presumably accompanied by a change in the Na and K content of the glands; since the amount of Na found in the diluted digest increased from $0{\cdot}29 \pm 0{\cdot}010$ in controls ($n = 12$) to $0{\cdot}43 \pm 0{\cdot}048$ m-equiv/l. ($n = 10$), while that of K decreased from $0{\cdot}12 \pm 0{\cdot}002$ in controls ($n = 12$) to $0{\cdot}07 \pm 0{\cdot}004$ m-equiv/l. ($n = 9$). The effects of ouabain on both the release of oxytocin and vasopressin as well as on the Na and K content of the pituitary glands were reversed by subsequent addition of ATP (30 mM) to the incubation medium. ATP alone had no effect.

To see whether the action of ouabain was dependent on the presence of calcium ion, pituitary glands were incubated in a calcium-free 'Locke' solution in the presence of ouabain. The effect of the glucoside appeared to be reduced. Subsequent addition of $CaCl_2$ (2·2 mM) to the medium containing ouabain enhanced the release of both hormones to a level similar to that observed when ouabain had been added to a standard Locke solution (Fig. 3).

Release of hormones from glands of lactating female rats

Lactating rats, with average litters of nine, were killed 18 days after parturition; their pituitary glands were removed and incubated in the usual way, five at a time in 1·0 ml. Locke solution, for periods of 20 min. The rate of release of vasopressin was much the same as that in control adult animals ($11{\cdot}7 \pm 0{\cdot}53$ m-u./20 min/5 glands) (24) but that of oxytocin was significantly decreased ($15{\cdot}7 \pm 1{\cdot}27$ m-u./20 min/5 glands (28). During lactation there is a fall in the oxytocin content of the posterior lobe, but not in that of vasopressin (Dicker & Tyler, 1953*a*, *b*; Acher & Fromageot, 1956). The smaller amount of oxytocin present in the hypophysis of lactating animals may therefore have been the reason for the smaller amount of hormone released during incubation. This is supported by the observation that the ratio between the quantity of hormones present in the glands and the amount released during a period of 20 min incubation was much the same in control and in lactating animals (Table 1).

To see whether glands from female lactating rats reacted in the same manner as those from control adult rats the following series of experiments were performed.

(*a*) incubation in 'Locke' solution containing an excess concentration of potassium ion (KCl = 56 mM), first in the absence (four experiments) and second, in the presence of $CaCl_2$ (2·2 mM) (five experiments);

(*b*) incubation in a medium containing increasingly greater amounts of

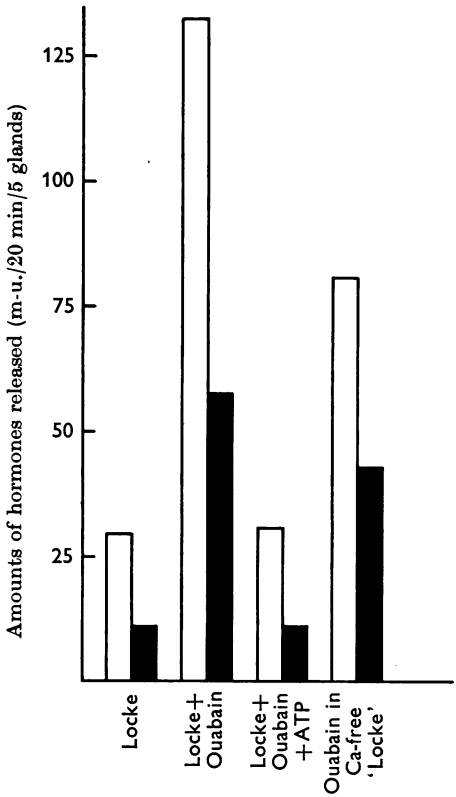

Fig. 3. Action of ouabain in the presence or absence of calcium ion on the release of vasopressin and oxytocin. White column: oxytocin; black column: vasopressin. Note that addition of ATP (30 mM) to ouabain reduces the amount of hormones released to a level similar to that of control.

TABLE 1. Relation between amounts of hormones in the gland and amounts of hormones released during incubation

	Vasopressin			Oxytocin		
	A Average content (m-u./5 glands)	*B* Average amounts released (m-u./ 20 min/5 glands)	*A*/*B*	*A* Average content (m-u./5 glands)	*B* Average amounts released (m-u./ 20 min/5 glands)	*A*/*B*
Adult rats, both sexes	4500	12·3	366	4600	28·7	160
Female rats, after 18 days lactating	4380	11·7	372	2350	15·7	150
New-born rats (24–48 hr)	40*	1·1*	36	14*	0·5*	35

The body weight of adult rats was 180–225 g.

Values for content and release are averages from twelve experiments.

* These values were calculated from results obtained from twenty pooled glands (see text).

$CaCl_2$ (varying from 2·2 to 8·8 mM) in the presence of an excess of KCl (56 mM) (twelve experiments);

(*c*) incubation in 'Locke' solution containing ouabain, first in the absence (four experiments), second in the presence of $CaCl_2$ (2·2 mM) (four experiments);

(*d*) incubation in a medium in which $CaCl_2$ had been substituted by $BaCl_2$ (2·5 μM) (six experiments).

But for the fact that the amount of oxytocin released in the control samples (14·4 ± 1·26 m-u./20 min/5 glands) was smaller than in non-lactating rats, changes in the rate of hormones released were similar to those described for non-lactating adult animals.

New-born rats

The amount of oxytocin and of vasopressin in the hypophysial glands of new-born is much smaller than that in adult animals (Dicker & Tyler, 1953*a*, *b*; Heller & Lederis, 1959). New-born animals and babies do not concentrate their urine as adults do (Heller, 1944). This has been attributed either to an immaturity of their kidneys (Dicker & Eggleton, 1960) or to a lack of response of the posterior pituitary gland to stimuli which in the adult produce a secretion of the hormones. The urine of neonates contains, however, appreciable amounts of an antidiuretic substance, presumably vasopressin, which do not appear to change with the state of hydration of the infants (Dicker & Eggleton, 1960). It was thought of interest therefore to see whether hypophysial glands of new-born rats would release *in vitro* the hormones in a way similar to that observed in adult animals. Rats aged 1, 8, 15 and 21 days were used. In view of the small hormone content of their hypophysis, twenty glands were pooled. They were incubated in 1·0 ml. of the standard Locke solution, in the usual way.

In twenty-one experiments, each using twenty whole hypophyses from 24 hr old rats, the amount of vasopressin released during a period of 20 min incubation was 4·5 ± 1·07 m-u./20 min/20 glands. This increased to 5·7 ± 0·91 (16) in 8-day-old rats and was as much as 24·8 ± 0·31 (6) m-u./20 min/20 glands in 21 day old animals. It can be calculated that the amount of vasopressin released by glands of 24 hr old rats, during periods of incubation of 20 min, represented about one tenth of that from adult animals (Table 1).

For technical reasons, it was not possible to estimate oxytocin and vasopressin in the same samples. The rate of release of oxytocin from pituitary glands of new-born animals was therefore investigated in a separate series of twenty-one experiments, pooling twenty glands each time. Oxytocin was found in the incubation medium of seven only out of the twenty-one experiments. The mean amount of oxytocin found in these

experiments was $1{\cdot}5 \pm 0{\cdot}42$ m-u./20 min/20 glands (7). In the other fourteen experiments no assayable amounts of oxytocin could be detected.

It will be seen from Table 1 that when the amount of hormones released was related to the actual hormone content of the glands, the output from pituitary glands of new-born animals was markedly greater than that from glands of adult animals.

Effect of increased concentration of potassium ion. In a series of twelve experiments, twenty glands from 24 hr old rats were incubated first in the standard Locke solution in the usual way and then transferred to a modified solution in which the KCl concentration had been increased 10-fold (56 mM), the concentrations of all other ions remaining unchanged. Vasopressin was estimated in six experiments, oxytocin in the remainder. In contrast with what had been observed for adult rats in none of these experiments did the increased concentration of KCl have an effect. The amounts of vasopressin released remained unchanged. Likewise for oxytocin which was present in three only of the control periods the amounts released remained unaffected.

Similar experiments were repeated using pituitary glands of neonates 8, 15 and 21 days old. Glands from animals 8 and 15 days old showed the same insensitivity to the stimulus of an excess of potassium ion as that observed for glands of new-born rats. When glands from neonates of 3 weeks of age were used, however, the increased concentration of potassium ion produced an enhanced release of oxytocin and vasopressin, qualitatively similar to that observed with glands from adult animals.

Action of ouabain. The effect of ouabain (0·5 mM) was tested on pituitary glands from new-born rats, in the presence of $CaCl_2$ (2·2 mM) and in its absence. Oxytocin was assayed in six experiments and vasopressin in six others. As the level of hormones released during the control period of incubation was so low, and as in the majority of cases the concentration of oxytocin was even too small to be assayed, it was not possible to ascertain the role of Ca ion alone in these experiments.

The effect of ouabain, however, was manifest in all experiments, whether $CaCl_2$ was present or absent. There was always a marked increase in the release of vasopressin. For oxytocin, in four experiments in which oxytocin had not been detected during the control period of incubation, measurable amounts of the hormone were found after addition of ouabain; and in the experiments where a low level of oxytocin had been detected before the addition of ouabain, the glycoside produced a marked increase of the release of oxytocin. In contrast with what had been observed with glands of adult rats, there was no difference between the effect observed with ouabain, when used in the presence or absence of $CaCl_2$ in the incubation medium.

DISCUSSION

Incubation of pituitary glands of adult rats, in a Locke solution at 37° C, resulted in a simultaneous release of oxytocin and vasopressin. The amount of oxytocin released during successive periods of 20 min was always greater than that of vasopressin. The basal rate of release of oxytocin and vasopressin from the pituitary glands of adult animals, including lactating females, appeared to be related to the amount of hormones present in the neural lobe. In all adult rats, irrespective of sex and of whether or not they were nursing their litter, the fraction of vasopressin released during a period of 20 min of incubation was of the order of 1/400 and that of oxytocin of about 1/150 of the hormone content of the gland (Table 1). In contrast, when pituitary glands of new-born animals were used, the amount of vasopressin released exceeded that of oxytocin. In many cases the amount of oxytocin released was too small to be assayed. This is consistent with previous observations (Dicker & Tyler, 1953*a*, *b*; Acher & Fromageot, 1956; Heller & Lederis, 1959) according to which there is more vasopressin than oxytocin in the hypophysial glands of new-born animals. The portions of vasopressin and oxytocin released during a period of 20 min incubation were of the order of 1/35 of the total hormone content of the gland. Thus, the proportion of vasopressin released by pituitary glands of new-born rats was significantly greater than that from adult glands (Table 1).

The neurosecretory cells of the pars nervosa possess electrical properties like ordinary neurones. They differ from ordinary neurones, however, in that their secretory products, instead of acting at the junction with other neurones, are transported by the blood stream and act on target organs remote from their site of origin. Electron microscope studies of the posterior pituitary gland have revealed in the cells the existence of two types of vesicles, some with a diameter of about 1500 Å, the neurosecretory vesicles, and others, with a diameter of the order of 200 Å, which resemble the synaptic vesicles of the cholinergic nerve terminals. The opacity of the neurosecretory vesicles does not appear to be uniform but to vary between those described as electron dense and those reported as clear. It is thought that the clearer or optically empty vesicles would correspond to vesicles which have released their hormone, though recent work by Daniel & Lederis (1965) has thrown some doubt on the correctness of this interpretation. Furthermore, it is not yet known whether each vesicle contains both hormones, and if so whether in equal or unequal amounts, or whether some vesicles contain one hormone only. Judging from experiments by Pardoe & Weatherall (1955), La Bella, Beaulieu & Reiffenstein (1962), Barer, Heller & Lederis (1963), the possibility that oxytocin

and vasopressin are contained in separate vesicles should be borne in mind.

If the total amount of hormones which can be extracted from the pars nervosa is contained in the neurosecretory vesicles, the hormone content of the posterior pituitary gland must be equal to that found in the vesicles. According to recent results obtained in using ultracentrifugation techniques between 70 and 80% only of the total hormone content can be found in the vesicles, the remainder being in the supernatant fluid (Barer *et al.* 1963). The presence of hormones in the cytoplasm, outside the vesicles, was confirmed by Daniel & Lederis (1965) who showed *in vivo* that after a strong stimulation of the neurohypophysis by anaesthesia and haemorrhage, the depletion of hormones from the gland was at the expense of the cytoplasmic fraction only, the hormone content of the vesicles remaining unaffected (Lederis, 1965).

The kinetics of the secretion of the hormones are not yet understood clearly. For instance, according to Cross & Harris (1952), 15–20 sec only elapse between the electrical stimulation of the supraoptic tract and milk ejection. As the time necessary for the hormone to reach the target organ has been estimated at 14 sec, the secretion of oxytocin into the blood vessels must be almost immediate, though it would have to cross several membranes; these, according to microscope investigations, are the plasma membrane of the nerve extremities, two basal membranes, one in contact with the nerve fibres, the other adjacent to the capillary membrane and finally the capillary endothelium itself (Denamur, 1965). The problem is complicated even further by the fact that the hormones are not freely in solution, but are bound to an inert protein, called neurophysin (Acher, Chauvet & Olivry, 1956) from which they have to be first freed. The neurophysin is not located entirely in the neurosecretory vesicles, some of it appearing to be in the cytoplasm of the cells. The freeing or 'elution' of the hormones from neurophysin can be achieved by slight variations of the pH of the complex (Ginsburg & Ireland, 1965) or by addition of calcium (Smith & Thorn, 1965; Thorn, 1965).

According to Douglas & Poisner (1964*a*, *b*) the release of the hormones would follow the entry of calcium ion into the neurosecretory cells after depolarization of their membranes by potassium. Since isolated neurosecretory vesicles appear to be insensitive to the stimulation of an excess potassium in the presence of calcium (Daniel & Lederis, 1963), the mechanism of 'stimulus secretion coupling' suggested by Douglas & Rubin (1963) could apply only to that portion of the hormones which is in the cytoplasm of the cell and not to that in the neurosecretory vesicles.

In the present series of experiments, one of the most effective stimuli for the release of both oxytocin and vasopressin from the hypophysis and

the only one which was effective on glands from both adult and new-born animals was ouabain. The greater release of both hormones when stimulated by ouabain was accompanied in the adult by an extrusion of potassium ion, and an influx of sodium suggesting that the enhanced release of oxytocin and vasopressin was associated with ionic shifts across the cell membranes. According to Whittam & Willis (1962), Opit & Chernock (1965) and Garrahan & Glynn (1965), there is evidence that the enzyme system known as 'Na^+ and K^+ dependent adenosine triphosphate' is closely associated with the active transport of sodium and potassium ions across cellular membranes. The inhibitory action of ouabain on this system would be explained by attachment of the glycoside on the protein enzyme which is itself membrane bound. Thus the action of ouabain would appear to be confined to the membrane of the neurosecretory cells. Experiments to see whether this cardiac glycoside can liberate oxytocin and vasopressin from isolated neurosecretory granules are in progress.

The importance of the role of the 'sodium pump' in the mechanism of release of the neurohypophysial hormones is further illustrated by experiments in which an increase of the calcium ion concentration in the incubation medium produced an enhanced release of both hormones. As the enzyme adenosine triphosphatase appears to be inhibited by calcium ion (Moe & Farah, 1965), it is likely that calcium ion may contribute to the release of the hormones. This view is consistent with the observation that the rate of hormone release was at its lowest when glands were incubated in a calcium-free medium.

Of far greater importance is the role that calcium plays after it has penetrated into the cells, especially when there is an excess of potassium in the incubation medium (Douglas & Poisner, 1964*b*). It has been shown by Douglas & Poisner (1964*a*) for vasopressin and confirmed in the present investigation for both oxytocin and vasopressin that an excess of potassium, in the absence of calcium ion, decreases the rate of output of the hormones below the level of controls. This suggests that the depolarization of the cell membrane will lead to an enhanced release of the hormones only when calcium can penetrate into the cell. The uptake of calcium ion by the hypophysial cells has been demonstrated by Douglas & Poisner (1964*b*). Once the calcium ion is in the cell, it will release oxytocin and vasopressin from its binding to neurophysin and allow the hormones to diffuse into the incubation medium (Thorn, 1965). This is the situation in pituitary glands of adult rats. In new-born rats, however, calcium ion in the presence of an excess of potassium did not increase the release of the hormones. It is suggested that the inability of calcium to produce the enhanced release of vasopressin and oxytocin in neonates is due to the fact that the hormones are not bound to neurophysin as in adults. There are a

series of observations which support this hypothesis. According to histologists, Gomori-stainable material observed in the posterior pituitary gland indicates the presence of neurophysin but not that of hormones. No trace of Gomori-stainable material has been found in pituitary glands of human foetuses younger than 23 weeks (Benirschke & McKay, 1953), though the presence of vasopressin has been shown in human embryos from the fourteenth week of uterine life onwards (Dicker & Tyler, 1953*b*). Similar observations have been made in the chick. The results of numerous investigations on the first appearance of Gomori-positive material in the neurohypophysis all agree that at birth the material is either absent or at best scanty, and that in the rat it does not appear before the end of the first week of life, and then only in the form of very fine dust-like granules which aggregate as the animal gets older (Bargman, 1949; Dawson, 1953; Scharrer, 1954; Green & von Breemen, 1955; Rodeck & Caesar, 1956; Rodeck, 1958). Observations showing that the urine of new-born animals and babies contains substantial amounts of antidiuretic activity, presumably of neurohypophysial origin (Dicker & Eggleton, 1960), or that larger amounts of oxytocin can be extracted by acetone from the pituitaries of rats aged up to about 7 days than from the glands of adult animals (Heller & Lederis, 1959) all suggest that the hormones are not bound in the same way as in the adult animal. Finally, in agreement with these views are the present findings that, proportionately to the hormone content of the gland, the amount of vasopressin released during incubation is greater in new-born than in adult rats. There is thus a considerable body of evidence which supports the hypothesis that in new-born animals there is either no neurophysin, or if it is present that its binding capacity for the hormones is different from that observed in adults. In either case, the entry of calcium into the neurosecretory cell, following depolarization by potassium ion, could not exert its competitive action for neurophysin and so release the hormones. This then may be the explanation why the 'stimulus-secretion coupling' system (Douglas & Poisner, 1964*a*) does not operate in the new-born animal.

It is now possible to offer an explanation of the mechanism of secretion of hormones in the posterior pituitary glands. It is suggested that the 'stimulus-secretion coupling' can operate only for the release of hormones contained in the cytoplasm of the cells, but not for the hormones located in the neurosecretory vesicles. In glands of adult rats the stimulus starts with the depolarization of the membrane followed by the uptake of calcium ion by the cell. In the cell, calcium ion competes for neurophysin and so releases the hormones from their attachment to the protein carrier. In pituitary glands of new-born animals, however, even if calcium can enter the cell, it cannot liberate the hormones, either because there is no neurophysin

present or because its binding capacity is different from that in glands of adult rats. The actual diffusion of the hormones across the cell membrane would then be achieved or be accompanied by a shift of ions, similar to that observed after addition of ouabain to the incubation medium of pituitary glands from either adult or new-born rats. Thus, whereas in the hypophysis of adult animals, the release of hormones involves their liberation from a protein carrier before their secretion, in glands of new-born animals the release of hormones may be explained simply by their diffusion across the cell membrane. This would account for the observations that the secretion of posterior pituitary glands of neonates does not appear to be influenced by exogenous stimuli in the same way as neurohypophysial glands of adults.

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