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STIMULI FOR THE RELEASE OF NEUROHYPOPHYSIAL HORMONES

BY

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A wide variety of stimuli has been described which release neurohypophysial hormones under experimental conditions *in vivo* (see the reviews of Denamur, 1965, and Heller & Ginsburg, 1966). In much of the work reviewed, however, the release of vasopressin and/or oxytocin was assessed by its effect on a target organ in the stimulated animal. Under these conditions there may be some doubt as to whether the effect of the stimulus on the target organ is entirely caused by vasopressin or oxytocin. Other substances, released by the same stimulus, might either mask the effect of the released hormone, imitate its effect or block its release. For example, it has been shown that adrenaline, which might be released in addition to the neurohypophysial hormones by some of the stimuli used, may mask the effects of oxytocin on the mammary gland and inhibit the release of oxytocin from the neurohypophysis (Cross, 1953, 1955a, b). It has also been shown (O'Connor & Verney, 1945 ; Verney, 1947 ; Duke & Pickford, 1951) that adrenaline can inhibit the release of vasopressin and that it can itself cause an antidiuresis. Other endogenous substances may also cause an antidiuresis, for example, bradykinin, angiotensin and substance P (Bisset & Lewis, 1962) but their activity is probably chiefly the result of cardiovascular effects. In addition many of the stimuli used to release vasopressin may cause fluctuations in blood pressure and this is likely to affect the rate of urine flow.

The purpose of the present investigation was to find several experimental stimuli which would increase the antidiuretic activity of external jugular blood of rats and to compare their effectiveness. After several effective stimuli had been found it was decided to carry out further experiments in which blood samples were extracted by a method devised to extract oxytocin and vasopressin from blood and to assay the extracts both for antidiuretic and for milk ejection activity before and after incubation with sodium thioglycollate.

In addition to showing which of the stimuli were the most effective under the conditions used, these experiments could be expected to provide evidence as to whether or not vasopressin and oxytocin can be released independently. If the proportion of vasopressin to oxytocin released by one stimulus differed from that released by another, the implication would be that the two hormones can be released independently.

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The choice of anaesthetic for this type of experiment presents a problem. An anaesthetic is desirable because the emotional disturbance which would almost certainly accompany blood collection from an unanaesthetized animal is likely to cause a release of vasopressin (Rydin & Verney, 1938). In addition anaesthesia makes blood collection and the stimulation of vasopressin release much easier. Nearly all anaesthetics release vasopressin (Heller, 1951), however, so that it is difficult to avoid an artificially raised initial blood concentration of vasopressin. Ether was chosen as the anaesthetic in this series of experiments because, although it causes some release of vasopressin (Ames & van Dyke, 1952), it does not prevent a further release of vasopressin after further stimulation (Ginsburg & Brown, 1956). In addition, atropine was given to prevent the inhibition of the heart and depression of the blood pressure which would have otherwise accompanied the injections of acetylcholine and carbachol, which were to be used as stimuli for vasopressin release. Beleslin, Bisset, Haldar & Polak showed in 1967 that a fall in blood pressure would itself release vasopressin. The use of atropine has the additional advantage that it prevents the formation of tracheal and bronchial exudate which occurs when ether is used as an anaesthetic. It has been shown by several workers that atropine does not prevent the release of vasopressin (Pickford, 1939; Fang, Liu & Wang, 1962; Mills & Wang, 1964b) although de Wied & Laszlo (1967) reported that it may do so in certain circumstances.

METHODS

Male albino rats of approximately 150 g body weight were given atropine sulphate 1 mg/10 g subcutaneously and after about 10 min were anaesthetized with ether. The right femoral vein was cannulated for the injection of heparin and the right external jugular vein was cannulated with a cannula of larger diameter to collect blood from the head. The right common carotid artery was then cannulated in experiments in which intracarotid injections were to be given, and ligatured in the other experiments. The right vagus nerve was ligatured and severed caudal to the ligature in experiments involving vagal stimulation.

All blood samples were taken through polythene cannulae into polythene tubes and injected into the assay animals with plastic syringes to prevent the formation of interfering substances which can occur if blood is stored in glass containers (Bisset & Walker, 1957). Assays of the activity of whole blood samples were started immediately after collection and a four point assay could be completed in about 1 hr. No decrease in the activity of the blood samples was observed during the course of the assays so it was assumed that for practical purposes no degradation of antidiuretic hormone occurred during this time. This assumption has been made by other workers (Ginsburg & Heller, 1953; Ginsburg & Brown, 1956). In experiments in which neurohypophysial hormones were extracted from blood, the extracts were made immediately after the completion of the blood collection. The procedure for the extraction of hormone from blood was that described by Bisset, Hilton & Poisner (1967). Briefly this consists of the removal of the blood cells by centrifugation and the precipitation of the plasma proteins with absolute alcohol. The inactivation of the extracts by sodium thioglycollate was carried out by the technique of Vogt (1953).

The antidiuretic assays were performed on male albino rats using a method derived from that of Jeffers, Livezey & Austin (1942); the urine flow was recorded and the water load maintained by the apparatus described by Dyball, Lane & Morris (1966). Four point (2+2) assays were performed routinely.

Milk ejection assays were performed on lactating guinea-pigs by the technique described by Tindal & Yokoyama (1962). Eight point assays (2 blocks of 2+2) were performed routinely.

The standard hormone preparation used for antidiuretic assays was Tonephin (arginine vasopressin) kindly supplied by Hoechst Ltd. and that for the milk ejection assays was Syntocinon (Sandoz).

RESULTS

1. *Effect of haemorrhage on the blood concentration of vasopressin*

The stimulus of haemorrhage was given by withdrawing, from the external jugular vein, 6 ml. of blood per 150 g body weight in three 2 ml. samples after the injection of heparin 1 i.u./g into the femoral vein. Ginsburg & Smith (1959) have shown that a considerable quantity of blood must be withdrawn before a detectable amount of antidiuretic hormone (ADH) appears in external jugular blood. The first 2 ml. was therefore treated as the "before haemorrhage" sample and the third 2 ml. was treated as the "after haemorrhage" sample. Table 1A shows the results of seven experiments; no antidiuretic activity could be detected in the first 2 ml. sample, confirming the findings of Ginsburg & Smith but, after severe haemorrhage, a marked increase occurred in the antidiuretic activity of external jugular blood.

TABLE 1A

EFFECT OF HAEMORRHAGE ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD (EXPRESSED IN μ-u./ml. OF ARGININE VASOPRESSIN) FROM RATS PRETREATED WITH ATROPINE

Before haemorrhage	After haemorrhage
<125	593
<125	373
<125	500
<125	880
<125	555
<125	3,337
<125	793

Table 1B shows the results of five experiments carried out in the same way but without the use of atropine. It can be seen that with the stimulus of haemorrhage at least, atropine does not substantially reduce vasopressin release. (The mean concentration of vasopressin in the blood after haemorrhage is 1238 without atropine and 1004 with atropine.) Atropine does not therefore always inhibit vasopressin release, although it may do so in response to some stimuli, so that its use in experiments on vasopressin release is permissible.

TABLE 1B

EFFECT OF HAEMORRHAGE ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD (EXPRESSED IN μ-u./ml. OF ARGININE VASOPRESSIN) FROM RATS NOT PRETREATED WITH ATROPINE

Before haemorrhage	After haemorrhage
<125	2,700
<125	1,182
<125	686
<125	890
<125	730

2. *Effect of the intracarotid injection of acetylcholine (0.66 mg/kg) into the right common carotid artery*

After the injection of heparin into the femoral vein, 1 ml. of blood was taken from the right external jugular vein. Acetylcholine (0.66 mg/kg dissolved in 0.2 ml. of physiological saline) was then injected, slowly, through a cranially directed cannula in the right

common carotid artery and washed in with 0.05 ml. of 0.85% sodium chloride solution. A second blood sample of 1 ml. was then taken from the external jugular vein (approximately 30 sec after the injection) and both samples were assayed for antidiuretic activity. Table 2 shows that an increase in blood ADH concentration was detected in three of the seven experiments only. Because no antidiuretic activity was detected in the first 2 ml. sample of blood taken in the experiments using haemorrhage as a stimulus (see Table 1), it was assumed that any activity found in the second 1 ml. sample in these experiments resulted from the acetylcholine injection.

TABLE 2

EFFECT ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD OF THE INJECTION OF ACETYLCHOLINE (0·66 mg/kg) INTO THE RIGHT COMMON CAROTID ARTERY

Results are expressed as in Table 1.

Before injection	After injection
<125	220
250	630
<125	<125
<125	<125
<125	<125
<125	161
<125	<125

The lack of efficacy of a comparatively high dose of acetylcholine may have been caused by a failure of the acetylcholine to reach the acetylcholine-sensitive areas in the central nervous system (probably in the supraoptic and paraventricular nuclei) activation of which can release ADH (Pickford, 1939, 1947; Duke & Pickford, 1951). If so, this could have been the result of anatomical differences in the blood supply of the brain between the rat and the dog because Pickford and her colleagues used dogs. Alternatively the acetylcholine may have been inactivated, enzymically, before it could reach the nuclei. Accordingly a further series of experiments was performed with carbachol which it was hoped would act as a stable form of acetylcholine. Table 3 shows that the second suggestion is the more likely because, in four out of five experiments, release of ADH occurred after the injection of carbachol.

TABLE 3

EFFECT ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD OF THE INJECTION OF CARBACHOL (0·66 mg/kg) INTO THE COMMON CAROTID ARTERY

Results are expressed as in Table 1.

Before injection	After injection
<100	<100
<50	167
76	391
48	259
113	272

3. *Effect of the intracarotid injection of a hypertonic sodium chloride solution on the antidiuretic activity of jugular blood*

The effect of the injection of a hypertonic sodium chloride solution (0.94 ml. of a 5% solution) into the right common carotid artery was compared with that of an injection of the same volume of an 0.85% sodium chloride solution. Table 4 shows that 0.94 ml. of 5% sodium chloride solution (injected at the rate of 0.6 ml./min) was an ineffective stimulus for the release of vasopressin.

TABLE 4

EFFECT ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD OF THE INTRACAROTID INJECTION OF (a) 0·94 ml. OF 5% SODIUM CHLORIDE SOLUTION COMPARED WITH THAT OF (b) THE SAME VOLUME OF 0·85% SODIUM CHLORIDE SOLUTION

Results expressed as in Table 1.

(a)		(b)	
Before injection	After injection	Before injection	After injection
<25	<25	103	200
<25	39	<50	<50
26	4	<50	<10
119	54	210	115
76	151	47	330

4. *Effect of stimulation of the central end of a severed vagus nerve*

Table 5 shows the effect on the antidiuretic activity of jugular blood of electrical stimulation of the central end of the severed right vagus nerve for 30 sec with square waves of 15 V, 2 msec duration and 100 c/s. (This resulted in a cessation of breathing presumably resulting from stimulation of afferent fibres involved in the Hering-Breuer reflex, so that a break of 2 sec was made between the fifteenth and seventeenth seconds to avoid the anoxia which would have occurred after 30 sec of apnoea.) This stimulus caused a considerable increase in the antidiuretic activity of external jugular blood. (The second blood sample was taken approximately 30 sec after the end of the stimulus.) A similar assumption was made in this series of experiments as in those described in section 2. Because 2 ml. of blood could be taken in the experiments in which haemorrhage was used as a stimulus without the appearance of a detectable rise in its antidiuretic activity, it was assumed that any activity in the second 1 ml. of blood was due to the vagal stimulation.

TABLE 5

EFFECTS OF ELECTRICAL STIMULATION (AT 15 V WITH SQUARE WAVES OF 2 msec DURATION AND 100 c/s) OF THE CENTRAL END OF THE SEVERED RIGHT VAGUS NERVE ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD

Results expressed as in Table 1.

Before stimulation	After stimulation
<200	2,457
<50	665
<50	238
<50	623
<200	1,366
364	1,888
<200	434
<100	384

5. *Effect of injecting calcium chloride*

Injection of 0.94 ml. of 0.1 M calcium chloride solution (approximately 25 mg Ca^{++}/kg) at a rate of 0.6 ml./min into the right femoral vein caused some release of antidiuretic activity. The effect was much more marked if the injection was made into the right common carotid artery (see Table 6). (Table 4 shows that the intracarotid injection of 0.94 ml. of 0.85% sodium chloride solution does not cause a marked rise in the blood antidiuretic activity.)

TABLE 6

EFFECT ON THE ANTIDIURETIC ACTIVITY OF EXTERNAL JUGULAR BLOOD OF THE INJECTION OF 0·94 ml. OF 0·1 M CALCIUM CHLORIDE SOLUTION (/150 g) (a) INTO THE RIGHT FEMORAL VEIN AND (b) INTO THE RIGHT COMMON CAROTID ARTERY

Results are expressed as in Table 1.

(a)		(b)	
Before injection	After injection	Before injection	After injection
<100	100	50	580
71	118	<100	>400
<100	232	<100	215
50	<50	32	537
108	721	<100	250
<100	38	<100	444
		63	4,029
		309	5,380
		249	325
		388	720

6. *Effect of haemorrhage, vagal stimulation and the intracarotid injection of calcium chloride on the plasma concentration of both vasopressin and oxytocin*

After the initial experiments had been performed in which whole blood was assayed directly for antidiuretic activity, the three most effective stimuli for the release of antidiuretic activity were further investigated. Blood samples obtained from four experiments with vagal stimulation or calcium chloride injection and three after haemorrhage were pooled and extracted by the method of Bisset, Hilton & Poisner (1967). The extracts were then assayed for both antidiuretic and milk ejecting activity. In one experiment in each group the extracts were incubated with sodium thioglycollate and then re-assayed. Table 7 shows that each stimulus caused an increase in both milk ejecting and antidiuretic activity in the plasma and that this activity could be very much reduced by incubation with sodium thioglycollate. In two control experiments in which 0.85% sodium chloride solution was injected instead of 0.1 M calcium chloride, no increase of plasma concentration of ADH or oxytocin occurred.

TABLE 7

EFFECT OF HAEMORRHAGE, VAGAL STIMULATION, THE INTRACAROTID INJECTION OF 0·94 ml. OF 0·1 M CALCIUM CHLORIDE AND 0·85% SODIUM CHLORIDE ON THE ANTIDIURETIC AND MILK EJECTING ACTIVITY OF PLASMA FROM BLOOD SAMPLES TAKEN FROM THE EXTERNAL JUGULAR VEIN APPROXIMATELY 30 SEC AFTER STIMULATION

In each of the experiments the results are expressed in terms of μ-u./ml. arginine vasopressin or synthetic oxytocin.

* Experiments in which the extracts were incubated with sodium thioglycollate; the figures in brackets indicate the remaining level of activity.

	Antidiuretic activity in plasma		Milk ejection activity in plasma		Milk ejection activity after stimulation expressed as a % of antidiuretic activity
Stimulus	Before stimulus	After stimulus	Before stimulus	After stimulus	
Haemorrhage	222	4,734	220* (<250)	1,119* (289)	24
	171	5,241	170	1,592	30
	218* (<100)	3,025* (<100)	223	799	26
Vagal	135	1,404	471	927	66
stimulation	71* (<100)	777* (<100)	<100	534	69
	56	712	182* (<167)	460* (<167)	65
0·1 M Calcium	164	1,068	176	354	33
chloride	47* (<50)	534* (<50)	412	853	160
	203	383	217* (<83)	446* (<94)	116
	21	86	193	278	323
0·85% Sodium	126	119	285	248	—
chloride	172	336	181	120	—

DISCUSSION

Several authors have demonstrated a considerable release of vasopressin after haemorrhage (for example, Ginsburg & Brown, 1956) and the results reported here confirm their results (see Table 1). Table 2 shows that under the conditions used in the present series of experiments, an injection of acetylcholine into a common carotid artery did not invariably cause a detectable rise in the blood concentration of antidiuretic activity. In three of the seven experiments a rise occurred, so that it is possible that in the other experiments there was a release of hormone but that it was not large enough to be demonstrated. These results, however, seem to be to some extent in contrast to those of Pickford and her co-workers (Pickford, 1939, 1947; Duke & Pickford, 1951) who invariably obtained an antidiuresis after this stimulus in dogs. The results shown in Table 3 suggest that the failure of acetylcholine to cause a release of antidiuretic activity in rats may have been the result of its very rapid breakdown after injection. Probably an insufficient quantity of acetylcholine reached the supraoptic and paraventricular nuclei to cause a detectable release of ADH, because carbachol released antidiuretic hormone more consistently.

The failure of intracarotid injections of a hypertonic solution of sodium chloride to raise the blood concentration of antidiuretic activity (see Table 4) is more puzzling because many authors have observed a release of vasopressin by this stimulus (see Heller & Ginsburg, 1966). The stimulus may have been too weak but nearly 1 ml. of 5% sodium chloride must be a fairly severe stimulus to a 150 g rat. A more likely explanation is perhaps that the second sample of blood (which was taken approximately 30 sec after the injection had been completed) was taken before the stimulus had exerted its full effect, for in his positive experiments de Wied (1960) waited 15 min before taking a second sample of blood and Ames, Moore & van Dyke (1950) waited 30 min. In addition, de Wied & Laszlo (1967) showed that atropine reduced the release of vasopressin by hypertonic saline in rats by 50% and all the animals used in these experiments were treated with atropine. On the other hand, there have been other reports of the ineffectiveness of hypertonic stimulation in releasing vasopressin in rats (Ginsburg & Brown, 1957) and these authors did not pretreat their rats with atropine. Yet another possibility is that both in the present experiments and in those of Ginsburg & Brown (1957) activation of the sympathetico-adrenal mechanism inhibited the expected release of vasopressin.

Table 5 shows that electrical stimulation of the central end of a severed vagus nerve released ADH which confirms the results of Chang, Chia, Huang & Lim (1939) and Mills & Wang (1964a, b). Table 6 shows that intravenous injections of calcium chloride cause a release of vasopressin and that this stimulus is more effective if the calcium chloride is given by the intracarotid route. This confirms the results of Thorn, Smith & Skadhauge (1965) who showed that in water-loaded rats the injection of calcium chloride causes an antidiuresis.

All these results are open to the objection that the antidiuretic activity observed in the blood samples taken after stimulation may not have been caused by vasopressin. The log dose-response lines for the antidiuretic effects of the blood samples and for the vasopressin standard were similar, however, and the difference in slope between the log dose-response lines for the standard and the antidiuretic activity in the blood samples

on thirty-seven separate occasions was not significantly different from zero ($P = >0.3$). In addition, Table 7 shows that the activity extracted from samples of blood taken before and after the three most effective stimuli (haemorrhage, vagal stimulation and injection of calcium chloride) was almost completely abolished by incubation with sodium thioglycollate.

Milk ejection assays were also performed on extracts of blood samples taken before and after the stimuli of haemorrhage, vagal stimulation and injection of calcium chloride and in each case (see Table 7) both milk ejection activity and antidiuretic activity were increased after stimulation. Milk ejection activity after stimulation expressed as a percentage of the antidiuretic activity (see Table 7) was lowest after stimulation by haemorrhage (24%, 30% and 26% in three experiments). Assuming that only 75% of the milk ejection activity was caused by neurohypophysial hormone (the smallest proportion which could be inactivated by incubation with sodium thioglycollate) the proportion of milk ejection to antidiuretic activity was 18%, 23% and 20% respectively. For estimations of the milk ejecting activity of arginine vasopressin, standard gave a mean of 4.6% $\pm$0.22 (S.E.). In addition, 95% fiducial limits were calculated for each of the assays on which these estimates were made according to Gaddum (1953) and the highest value between these limits was 8%. Other recent estimates of the milk ejection activity of vasopressin have been higher (13.6%, Clark & Rocha e Silva, 1966; 8%, Bisset, Hilton & Poisner, 1967) but it is unlikely that all the milk ejection activity in the blood samples taken after haemorrhage was the result of the intrinsic milk ejecting activity of vasopressin. This finding is in contrast to the findings of Beleslin, Bisset, Haldar & Polak (1967) who could not detect any release of oxytocin by haemorrhage in cats but the difference may be explicable in terms of species differences and the different anaesthetic used (Beleslin *et al.* used pentobarbitone anaesthesia).

Ginsburg & Smith (1959) failed to detect release of oxytocin by haemorrhage in rats but here the difference may have been because of the less severe stimulus used by these workers (Ginsburg & Smith took 2.5 ml. of blood/100 g whereas 4 ml. were taken in the present experiments) and to the less sensitive assay methods then available. In view of the close anatomical proximity of the nervous pathways for the release of the two hormones, however, it is not unlikely that a powerful stimulus for the release of one hormone would release a small amount of the other. This does not exclude the possibility that one hormone can, in certain circumstances, be released without the other. Indeed, the different proportions of milk ejecting and antidiuretic activity released by the different stimuli used strongly implies that the two hormones are released by different mechanisms. This view would be consistent with the conclusions of Clark & Rocha e Silva (1966); Bisset *et al.* (1967) and Beleslin *et al.* (1967) that vasopressin and oxytocin can be released independently. They found that no release of oxytocin occurred after carotid occlusion, stimulation of certain areas of the hypothalamus or after haemorrhage but that each of these stimuli can release vasopressin.

SUMMARY

1. Six stimuli were investigated for their effectiveness in releasing vasopressin in rats anaesthetized with ether.

2. Severe haemorrhage, electrical stimulation of the central end of the severed right vagus nerve and the intracarotid injection of calcium chloride solution consistently raised the concentration of ADH in jugular blood. The intracarotid injection of acetylcholine (0.66 mg/kg) was not effective in every case but this was probably because of its rapid breakdown after injection because carbachol was a more reliable stimulus. The intracarotid injection of 5% sodium chloride solution under the experimental conditions used was not effective in releasing vasopressin.

3. Extracts of blood samples taken before stimulation by haemorrhage, vagal stimulation and calcium chloride injection were assayed for milk ejection and antidiuretic activity. These activities could be largely abolished by incubation with sodium thioglycollate and thus are likely to have been caused by posterior pituitary peptides.

4. Each of the three most effective stimuli released both vasopressin and oxytocin but in different proportions.

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