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The effect of metals on the S-S polypeptide receptor in depolarized rat uterus

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1. Metals potentiate the contractile effects of S-S polypeptides in depolarized rat uterus. Their order of potency is $Co^{++} \geqslant Co^{+++} \geqslant Mn^{++} \geqslant Ni^{++} \geqslant Mn^{+++} > Zn^{++} \geqslant Mg^{++} > Fe^{++} > Fe^{+++} \geqslant Ca^{++} > Be^{++} = Sr^{++} = Ba^{++} = Cu^{++} = O$.

2. S-S polypeptides with relatively weak oxytocic activity such as lys-vasopressin, arg-vasopressin and orn-vasopressin are strongly potentiated by metals while highly active polypeptides such as oxytocin are weakly potentiated.

3. Potentiation by metals was specific for S-S polypeptides ; other polypeptides (bradykinin, hypertensin) as well as acetylcholine and isoprenaline were unaffected.

4. Potentiation by metals occurs rapidly and is fully reversible. In all cases some activity was retained by S-S polypeptides even in the complete absence of metals.

5. A scheme which could account for the observed effects has been formulated. This assumes the formation of a ternary complex between receptor, metal and polypeptide leading to improved alignment between polypeptide and receptor.

6. Analogies are discussed between metal enzymes and the S-S polypeptide receptor for which the term metal receptor is proposed.

It has long been known that the response of the isolated uterus to S-S polypeptides is influenced by the magnesium content of the Ringer solution (Van Dyke & Hastings, 1928 ; Fraser, 1939 ; Stewart, 1949). Magnesium potentiates some S-S polypeptides while others are little affected, hence their relative activities depend on the amount of magnesium in the medium (Munsick, 1968). The actions of S-S polypeptides on vascular and intestinal smooth muscle and on myoepithelial cells are also potentiated by magnesium (Somlyo, Woo & Somlyo, 1966 ; Woo & Somlyo, 1967). More recently it has been found that manganese and cobalt can substitute for magnesium (Bentley, 1965 ; Somlyo *et al.*, 1966). Several authors have suggested that the effects of magnesium on S-S polypeptides are exerted at receptor level (Bentley, 1965 ; Somlyo *et al.*, 1966 ; Krejci & Polacek, 1968).

The present paper deals with the action of metals on the response to S-S polypeptides in depolarized rat uterus. The S-S polypeptides stimulate this preparation (Evans, Schild & Thesleff, 1958) and their effects on it are potentiated in a graded

and reversible manner by metals. The S-S polypeptide receptor-metal system shows similarities with reversible metal-enzyme complexes (Vallee & Coleman, 1964) and various analogies between them will be discussed.

Methods

Excised uterus horns of rats weighing 185–205 g were suspended in jacketed isolated organ baths of 6 ml. volume. The jackets were perfused and their temperature was controlled at 30° C. Two horns were normally used in parallel. Tension was measured by Swema (SG4/3) strain gauge transducers recording on an ink-writing Grass polygraph. The transducers were attached to screw stands ; a vernier-type sliding scale attached to the transducers enabled length changes of the uterus to be read to 0·1 mm. Glassware and automatic assay apparatus were as described by Boura, Mongar & Schild, 1954.

Preparations were first immersed in Tyrode solution ; responses to increasing doses of acetylcholine (ACh) were recorded until maximal effects were obtained. Maximal forces varied between 2·5 and 6 g ; uteri giving larger maximal responses were considered unsuitable. Preparations were then transferred to potassium Ringer which produced a rapid contraction followed immediately by relaxation to a new elevated baseline. At this point the uterus was permitted to shorten while maintaining a baseline tension of 0·3–0·6 g ; magnification was increased about five-fold because of the smaller responses to drugs of the depolarized uterus (Fig. 1). In the course of experiments, the muscle tended to shorten further, but generally it became stabilized after 1–2 hr. Changes of bath fluid often induced in the depolarized uterus complicated sequences of relaxation and contraction requiring a certain amount of manœuvering to restore the initial tension.

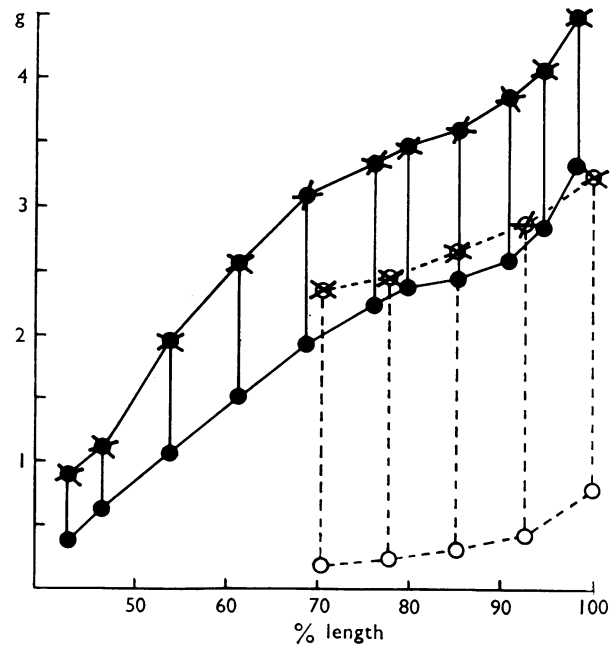

FIG. 1. Isometric contractions of a rat uterus horn in response to a maximal dose of oxytocin (×), 33 m-u./ml., at varying initial lengths and tensions: ●, K_2SO_4 Ringer, (1 mM Ca^{++}, 1 mM Mg^{++}) ; ○, Na_2SO_4 Ringer (same composition except that K^+ replaced by Na^+) ; 30° C.

Calcium-containing potassium sulphate Ringer was generally used as the depolarizing medium. Its composition was (mM): 105 K_2SO_4, 12 $KHCO_3$, 6 glucose, 1 KH_2PO_4 with either 0·1 or 1 $CaCl_2$. Osmosity (Wolf, 1966) of the solution was 152 and its pH with gentle bubbling of O_2—5% CO_2 was 7·4–7·6. Uteri probably remained viable for longer with the higher calcium Ringer, but the low calcium Ringer gave better initial responses and prevented the uterus from shortening progressively. In experiments with lysine vasopressin 1 mM $MgCl_2$ was usually added to the solution. Depolarized uteri responded to S-S polypeptides for several hours; when responses deteriorated they could generally be restored by brief exposure of the preparation to Tyrode solution followed by renewed depolarization (Schild, 1967). Other depolarizing solutions used were (mM): potassium sulphate-sucrose Ringer (74 K_2SO_4, 59 sucrose, 12 $KHCO_3$, 6 glucose, 1 $CaCl_2$) and potassium chloride Ringer (145 KCl, 12 $KHCO_3$, 6 glucose, 0·1 $CaCl_2$).

An important difference between drug effects in normal and depolarized uterus is that the latter are superimposed on an already shortened contractile element. In contrast to frog twitch muscle which responds to a maintained potassium-depolarization by a rapid contraction followed by complete relaxation (Hodgkin & Horowicz, 1960) rat uterus responds to maintained potassium-depolarization by contraction followed by incomplete relaxation; complete relaxation occurs only after repolarization. Figure 2 shows that a uterus kept in potassium sulphate Ringer remains considerably shortened, but it relaxes after being transferred to sodium sulphate Ringer. Figure 2 shows that replacing half the potassium by sodium does not cause relaxation. In these conditions the solution contains sufficient potassium sulphate to maintain depolarization (Kuriyama, 1963) so it can be concluded that relaxation is probably a consequence of repolarization rather than due to some special property of sodium ions.

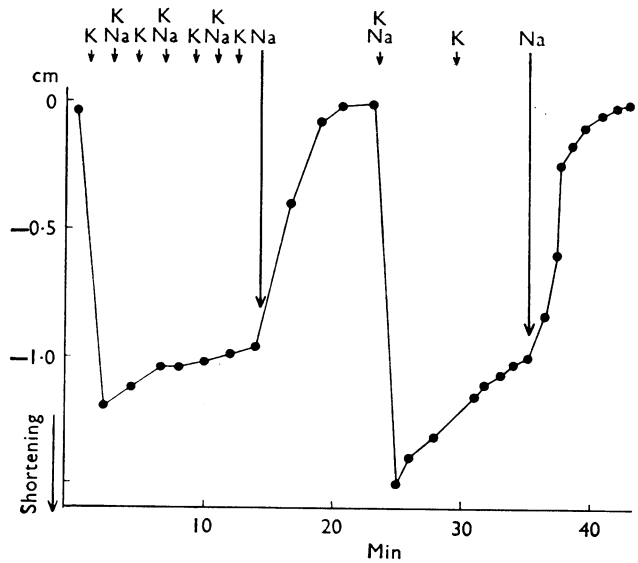

FIG. 2. Effect of K_2SO_4-Ringer (K), Na_2SO_4-Ringer (Na) and an equal mixture of both (K-Na) on length of uterus horn under 0·3 g load, 30° C. Ordinate: change of muscle length (cm); abscissa: time. Ringer solutions as in legend to Fig. 1.

Figure 1 shows the superimposed effect produced by an S-S polypeptide. Although the load-extension curve of the depolarized uterus resembles that of an electrically stimulated muscle (Schatzmann, 1964) a further contraction can be induced by oxytocin. The superimposed oxytocin response of the depolarized uterus is over a wide range independent of initial length and tension, but it is always considerably less than the response of the polarized muscle. In practice basal tensions were kept below those giving optimal drug effects in order to avoid damaging the preparation.

Drugs and reagents

The following were used. Lysine-8-vasopressin (10 u./ml. ampoules containing 5 mg chlorbutol), ornithine-8-vasopressin (5 u./ml. ampoules, no preservative), arginine vasotocin (0·1 mg/ml. with 1 mg methylparahydroxybenzoate), oxytocin (10 u./ml. ampoules with 5 mg chlorbutol); all synthetic compounds, Sandoz, kindly supplied by Dr. B. Berde. Arginine pitressin (arginine-8-vasopressin) powder containing 50 Pr.u./mg, Parke Davis, kindly supplied by Dr. A. C. Bratton. Hypertensin (angiotensin amide), Ciba; bradykinin, synthetic, Sandoz; isoprenaline sulphate, Burroughs Wellcome; acetylcholine iodide, British Drug Houses.

Beryllium sulphate, magnesium chloride and sulphate, manganous chloride and sulphate, cobaltous nitrate, sodium cobaltinitrite, nickel chloride and sulphate, ferrous sulphate, ferric chloride and nitrate, cupric chloride and sulphate, zinc sulphate, potassium chloride, sulphate, hydrogen carbonate, dihydrogen orthophosphate, strontium chloride, glucose, all A.R. reagents B.D.H. Other B.D.H. reagents: volumetric solution of ethylene-diaminetetraacetic acid (EDTA) N/50, and of calcium chloride M/1, ferrous chloride anhydrous. Barium chloride, suprapur, Merck. Acetylacetonates of manganese (II) pure, manganese (III) pure, cobalt (II) and cobalt (III) pure; cobalt trifluoride; all Koch-Light. Hexamminocobaltic chloride, laboratory reagent, Hopkin and Williams. Sodium cobalticarbonate kindly supplied by Dr. Bosnich. Distilled water deionized through Permutit.

Results

Effect of magnesium on vasopressin responses

Magnesium potentiates the contractile effect of lysine vasopressin in the depolarized uterus. Figure 3 shows an experiment in which the muscle was immersed in potassium sulphate Ringer containing 1 mM Ca^{++}; when 1 mM Mg^{++} was added to the bath the effects of vasopressin were potentiated about three-fold in terms of equivalent doses. Two sequences in reversed order on paired horns of the same uterus are shown; the action of magnesium is seen to be fully reversible.

Magnesium invariably potentiated lysine vasopressin but the degree of potentiation varied; it was generally greater than in Fig. 3, averaging about ten-fold in a series of 2 + 2 assays (Table 1). Magnesium had similar potentiating activity whether the solution contained 1 mM or 0·1 mM Ca^{++} (Table 1). In the complete absence of Ca^{++}, vasopressin, like other stimulants of depolarized smooth muscle, is inactive (Robertson, 1960; Durbin & Jenkinson, 1961; Edman & Schild, 1962). In these conditions magnesium cannot substitute for calcium.

A typical log dose-response curve for magnesium in depolarized rat uterus is shown in Fig. 4. The bath fluid contained 0.1 mM Ca^{++} throughout while the

concentration of Mg^{++} was varied from 5×10^{-5} to 5×10^{-3}M; the dose of lysine vasopressin was 10 m-u/ml. The responses are graded increasing to a maximum and subsequently decreasing with higher magnesium concentration.

It seemed possible that magnesium is an absolute requirement for the vasopressin receptor and that effects in the absence of magnesium in solution might be due to some magnesium remaining attached to the receptor. To test this possibility, a uterus horn was immersed in Ca-free K_2SO_4 Ringer containing 1 mM EDTA; this chelating agent has high affinity not only for calcium but also for magnesium (Ringbom, 1963) and it might thus be expected to remove both metals from superficial structures. It was found, however, that when calcium was applied again after 20 min exposure to EDTA the earlier response to vasopressin was restored. In other experiments depolarized uterus was immersed for relatively long periods (30–60 min) in magnesium-free solution. During this period the sensitivity to vasopressin declined progressively after an initial phase of rapid decline, but when magnesium was subsequently reintroduced the original sensitivity was restored.

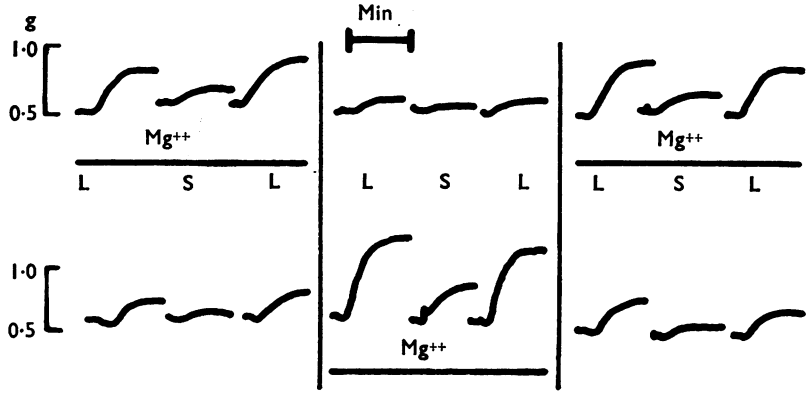

FIG. 3. Reversible potentiation by 1 mM Mg^{++} of responses to 0·3 (S) and 1 (L) m-u./ml. lysine vasopressin. Paired horns of rat uterus. K_2SO_4-sucrose-Ringer, 1 mM Ca^{++}. 30° C.

TABLE 1. *Potentiation of lysine vasopressin by magnesium in different experiments.*

Uterus in K_2SO_4-Ringer (30° C)		
Ca^{++} (mM)	Mg^{++} (mM)	Log dose ratio* in absence and presence of Mg^{++}
1	1	0·52
1	1	1·3
1	1	1·1
1	1	1·08
1	1	0·75
1	1	1·36
1	1	0·82
0·1	1	0·84
0·1	1·7	1·02
0·1	1·7	1·06
		Mean 0·99

* After 10–30 min incubation with or without Mg^{++}.

These findings suggest that vasopressin receptors can function in the absence of magnesium and that magnesium lack does not inactivate them irreversibly. The evidence is not conclusive, however, because drastic measures for magnesium removal could not be applied to the living muscle ; furthermore even ostensibly magnesium-free solutions contain traces of magnesium which might affect the receptors.

Rate of onset and decay of magnesium potentiation

The potentiating effect of magnesium is rapidly established. Figure 5 shows the results of an experiment in which magnesium was added at different times before and after the administration of a standard dose of lysine vasopressin ; the latter was just enough to produce a threshold response in the absence of magnesium. Sequences were randomized, each point in Fig. 5 representing four–six determinations. Magnesium potentiated vasopressin after 10 sec preincubation and its potentiating effect was only slightly less, and not significantly so, after 10 sec than after 1 and 3 min preincubation. When magnesium was applied 1 and 3 min after the vasopressin (a procedure also used by Somlyo *et al.*, 1966, and Krejci *et al.*, 1968) a response was initiated as soon as the magnesium was added ; magnesium thus seems to act as soon as it reaches the receptors already in contact with vasopressin. The responses were, however, significantly reduced compared to the reversed order of administration. The reduction may be due to the short contact of magnesium with the receptors or to desensitization.

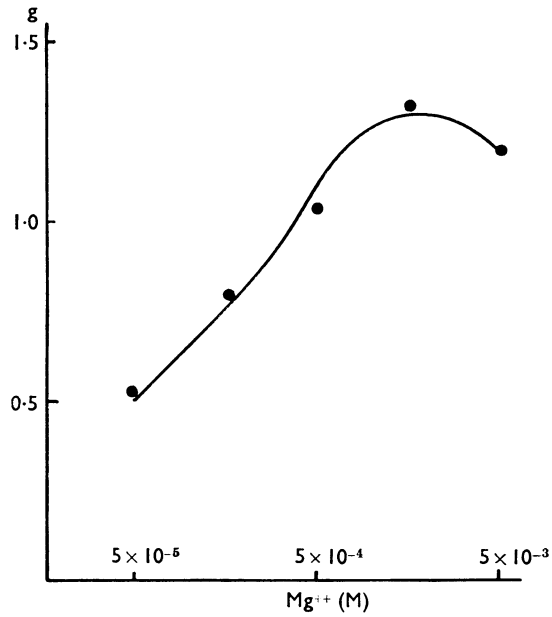

FIG. 4. Effect of different concentrations of Mg++ on response to lysine vasopressin 10 m-u./ml. Means of two results. Potassium sulphate Ringer, 0·1 mM Ca++. Isolated rat uterus, 30° C (this and subsequent tracings except for Fig. 7).

In other experiments, the rate of decay of magnesium potentiation was measured. A standard dose of vasopressin was administered after 3 min contact of the preparation with magnesium and at different times after washout. The potentiating effect declined exponentially with halftimes (at 30° C) varying between 0·5 and 2 min.

Effect of magnesium on other S-S polypeptides

Four S-S polypeptides other than lysine vasopressin were available for testing. Their structural differences from lysine vasopressin, as well as their degree of potentiation by magnesium are shown in Table 2.

Arginine vasopressin was strongly potentiated by magnesium, but less so than the lysine analogue, when the two were tested in the same preparation. Figure 6 shows the effect of 2 mM Mg^{++} on the two vasopressins ; the arginine analogue was

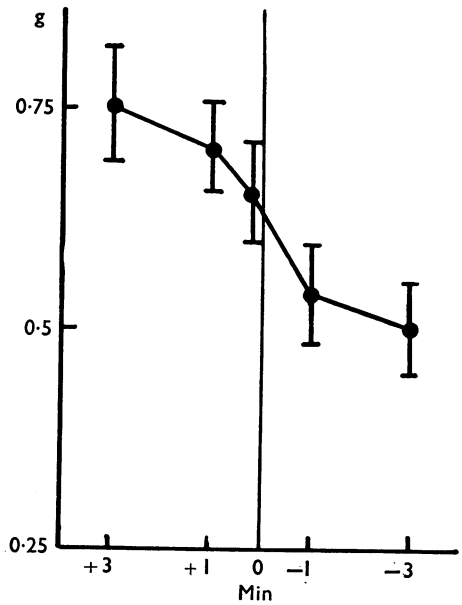

FIG. 5. Magnesium (1·7 mM) applied at different times before and after standard doses of lysine-vasopressin. Abscissa: min between Mg^{++} and vasopressin addition. Means and standard errors of four to six determinations.

TABLE 2. *Effect of Mg^{++} (1–2 mM) on response of depolarized rat uterus to S–S polypeptides*

	Difference from lysine vasopressin	Potentiation (log dose ratio) (ant=antagonism)
Arginine vasopressin	Arg^8 for Lys^8	0·42, 0·58, 1·24, 1·2, 0·92, 1·0, 1·0, 0·62 mean: 0·87
Ornithine vasopressin	Orn^8 for Lys^8	0·86, 1·48, 1·04, 1·54 mean: 1·23
Arginine vasotocin	Ile^3 for Phe^3 Arg^8 for Lys^8	0, 0·85, 0·94, ant, ant, 0·4, 0·38, 0·44
Oxytocin	Ile^3 for Phe^3 Leu^8 for Lys^8	0, 0, 0·42, 0·3, 0·38, 0·36, ant, ant

potentiated by a factor of 9 and the lysine analogue by a factor of 28, in both cases the log dose-response curves with and without magnesium were parallel. Ornithine vasopressin, structurally closely related to lysine vasopressin, also produced a parallel shift of the log dose-response curve giving a mean potentiation of seventeen-fold with 2 mM Mg^{++}.

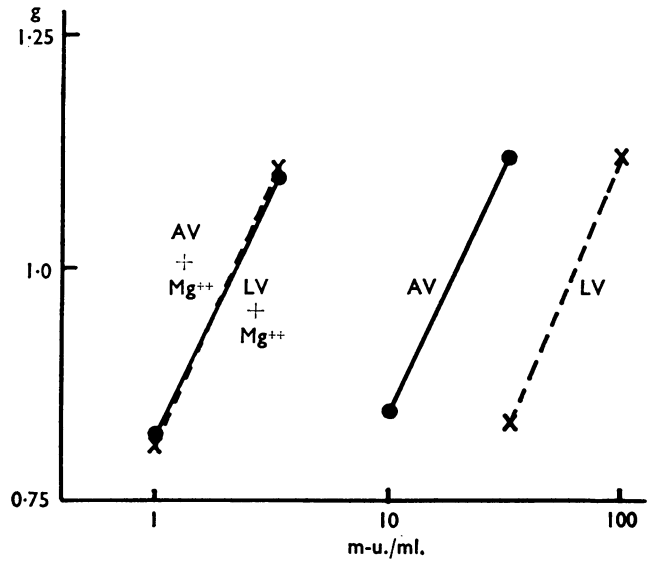

FIG. 6. Potentiation of (●——●) arg-vasopressin (AV) and (×– –×) lys-vasopressin (LV) by 2 mM Mg^{++}. Means of two responses. K_2SO_4-Ringer, 0·1 mM Ca^{++}.

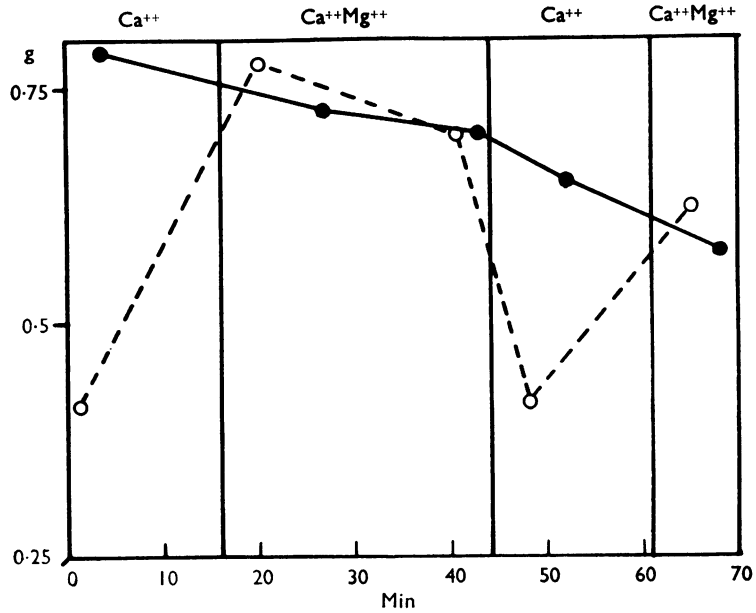

FIG. 7. Responses to (●——●) ACh (5×10^{-7}) and (○– –○) lysine vasopressin (1 m-u./ml.) in absence and presence of Mg^{++} (1 mM). K_2SO_4-sucrose-Ringer, 1 mM Ca^{++}. 37° C.

The other two S-S polypeptides tested, oxytocin and arginine vasotocin, show greater structural differences from lysine vasopressin as seen from Table 2. The effect of magnesium on these compounds varied from potentiation to no effect or depression. That oxytocin is less potentiated by magnesium than lysine vasopressin was also shown in another type of experiment in which the two polypeptides were assayed against each other in the presence and absence of 1 mM Mg^{++}. In 2+2 assays on depolarized rat uterus, oxytocin was invariably more active than lysine vasopressin, but the activity ratios of the two compounds changed in favour of vasopressin in the presence of magnesium. In six such experiments the mean change in the log activity ratio was $0{\cdot}82 \pm 0{\cdot}17$ (S.E.).

Effect of magnesium on drugs other than S-S polypeptides

Figure 7 shows a comparison between the effects of magnesium on acetylcholine and lysine vasopressin responses in depolarized rat uterus. The bath fluid (K_2SO_4-sucrose-Ringer) was changed repeatedly from calcium alone to calcium and magnesium (each in 1 mM concentration). Equiactive doses of the two drugs in the presence of magnesium produced widely different effects in its absence; the acetylcholine responses declined evenly in the course of the experiment, whereas the vasopressin responses declined when magnesium was omitted and recovered when it was added. Similar experiments with bradykinin and hypertensin II likewise failed to show a potentiating effect of magnesium. Magnesium seems to affect specifically S-S polypeptides.

Similar tests were done with isoprenaline, which relaxes the depolarized uterus (Schild, 1967) because of the possibility (Belleau, 1960) that metals may play a part in the interaction of beta-adrenergic drugs and their receptors. Magnesium (1 mM) was added intermittently as in the preceding experiment; graded relaxant effects were produced with isoprenaline and the concentration required to cause a selected response was estimated by interpolation from dose response curves. Successive results in an experiment of this kind on two horns of the same uterus are shown below; figures refer to log molar concentration of isoprenaline; results with magnesium are shown in italics:

Horn 1	9·21	*9·09*	9·13	*8·84*
Horn 2	*8·98*	8·85	*8·69*	8·59

The findings show no effect of magnesium on isoprenaline relaxation.

Potentiating effects of various metals

Besides magnesium a number of other metals potentiated the action of lysine vasopressin in the depolarized preparation. Ten metals have been investigated for their potentiating effect, three in two oxidation states. Some metals were studied in detail, others only cursorily in what may be regarded as a first survey of a vast field. Metals were chosen from group IIa of the periodic table to which magnesium belongs and from the first transition series which contains many of the elements known to activate enzymes.

Several of the metals were more active than magnesium and in order to obtain a quantitative estimate of their activity they were all compared with magnesium. Comparisons were carried out by 2+2 or 2+1 assays in which an appropriate concentration of metal was added to the bath 2–3 min before a standard dose of lysine

vasopressin. Vasopressin doses were chosen so as to produce small responses on their own capable of graded potentiation by metals; concentrations were in the range of 2·5–10 m-u./ml. lysine vasopressin. After each potentiated response, vasopressin alone was given until its basal response was restored; this usually happened after one or two doses, but some metals produced longer lasting effects requiring longer recovery periods. Many metals produced contractile or relaxant effects of their own and in these cases the length of the muscle was adjusted until basal tension was restored before adding vasopressin or other stimulants. Most assays were performed in potassium sulphate Ringer with either 0·1 or 1·0 mM Ca^{++}, but in testing calcium itself and strontium and barium, the sulphates of which have low solubility products, potassium chloride Ringer was used. Table 3 gives a summary of the potentiating activities of metals for lysine vasopressin in depolarized rat uterus, in terms of log activity ratios relative to magnesium.

Alkaline earth metals Be, Mg, Ca, Sr, Ba

Beryllium stands apart in this group because of its lack of ionization at pH 7 due to extensive hydrolysis (Gurd & Wilcox, 1956; Jones, 1964); it also is the group member of lowest atomic weight and smallest ionic crystal radius. Beryllium sulphate was tested at a concentration of 2 to 5×10^{-4}M and was found to be inactive, neither potentiating nor antagonizing vasopressin.

In contrast to beryllium, the elements magnesium, calcium, strontium and barium possess increasingly ionic character in that order (Durrant & Durrant, 1962) and their ionic crystal radii increase in the same order (Table 6). The potentiating effect of magnesium has already been mentioned; magnesium was employed as the sulphate and chloride; it usually produced some degree of initial contraction. Calcium, strontium, and barium were tested in potassium chloride Ringer containing 0·1 mM Ca^{++}. When added in 2 mM concentrations all three produced powerful contractions of the uterus requiring length adjustments. The three metals differed in their interaction with vasopressin, the contractile effect of which was slightly antagonized by strontium and barium but slightly potentiated by calcium. The potentiating activity of calcium for lysine vasopressin was of the order of only one-tenth that of magnesium (four experiments) but the effect was clearcut and

TABLE 3. *Log activity ratios of metals and magnesium in respect of potentiating activity for lysine vasopressin in depolarized uterus (depr=depressed)*

Group IIa		
Be^{++}	nil, nil, nil	
Ca^{++}	$-1{\cdot}0, -1{\cdot}0, <-1{\cdot}0, <-1{\cdot}0$	mean $=<-1{\cdot}0$
Sr^{++}	depr, depr	
Ba^{++}	depr, depr	
Transition Metals		
Mn^{++}	0·84, 0·96, 0·96, 0·94, 1·05, 0·98, 1·3, 1·0, 1·0	mean: 1·0
Mn^{+++}	0·8, 0·76, 0·7	mean: 0·75
Fe^{++}	$-0{\cdot}52, -0{\cdot}52, 0, -0{\cdot}5, -0{\cdot}25, 0, -0{\cdot}5$	mean: $-0{\cdot}33$
Fe^{+++}	$-1{\cdot}0, -1{\cdot}0$	mean: $-1{\cdot}0$
Co^{++}	1·1, 1·12, 1·2, 1·56, 1·02, 1·4, 1·4	mean: 1·26
Co^{+++}	1·18, 1·19	mean: 1·19
Ni^{++}	0·5, 0·96, 1·13, 1·08	mean: 0·92
Cu^{++}	depr, depr, depr	
Zn^{++}	0, 0, 0·13	mean: 0·04

apparently specific; calcium failed to potentiate acetylcholine under the same test conditions.

Metals of the first transition series: Mn, Fe, Co, Ni, Cu, Zn

Manganese, cobalt and nickel

These three metals produced powerful and fully reversible potentiating effects.

Manganese (II) was applied as the chloride, sulphate and acetylacetonate; the three forms seemed to be approximately equiactive. Graded potentiation of the effects of lysine vasopressin by 1·6 and 5×10^{-5}M manganous acetylacetonate are shown in Fig. 8. Unless very large doses were employed the potentiating effects of manganese reversed rapidly. The manganous ion was between 7 and 20 times more active than magnesium. Figure 9 shows the results of a comparative assay of manganous and magnesium chlorides; when the responses were plotted against log metal concentration parallel lines resulted. Figure 10 shows the specific nature of manganese potentiation. Graded doses of lysine vasopressin, oxytocin and hypertensin (II) were given first in the absence and then in the presence of manganous chloride; only vasopressin was potentiated by manganese, the other polypeptides were unaffected.

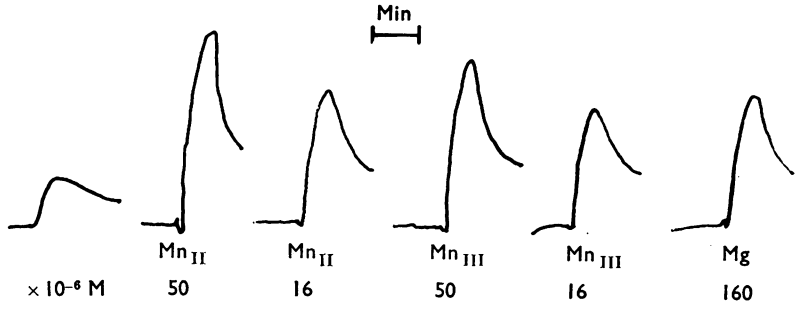

FIG. 8. Potentiation of lys-vasopressin 10 m-u./ml. by Mn (II) and Mn (III) acetylacetonates and Mg. First response lys-vasopressin alone. K_2SO_4-Ringer, 0·1 mM Ca++.

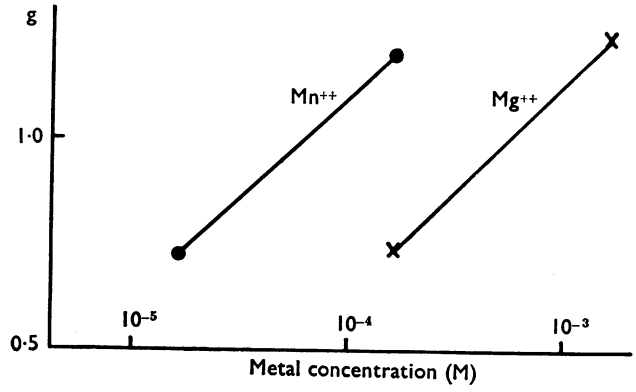

FIG. 9. Effect of Mn++ (●) and Mg++ (X) on response to lys-vasopressin (3 m-u./ml.). Means of two. Note parallel shift. K_2SO_4-Ringer, 0·1 mM Ca++.

Manganese (III) was tested as the acetylacetonate. A comparison of the acetylacetonates of manganese (II) and (III) is shown in Fig. 8. Both forms potentiated vasopressin in low concentrations, and although the divalent form was apparently more active this could be due to more complete ionization of its acetylacetonate.

Cobalt (II) was the most active of the transition metals, its mean activity being nearly twenty times that of magnesium. Cobalt (II) was employed as the chloride, sulphate and acetylacetonate ; the effect of 5×10^{-5}M cobaltous chloride in potentiating lysine vasopressin is shown in Fig. 11. Although the effects of cobalt were fully reversible they were more persistent than those of magnesium. Table 4 shows a comparison of the rate of decay of potentiation after equiactive doses of magnesium and cobaltous ion tested in the same preparation. Administration of a dose of metal was followed after 3 min by a dose of vasopressin ; further doses of vasopressin were given after washout. Half-times of recovery were calculated assuming exponential decay (preliminary experiments had shown that potentiation declined approximately exponentially). The rate of decay was significantly slower with Co^{++} than with Mg^{++} ($0{\cdot}01 < P < 0{\cdot}05$).

Difficulties were experienced in attempting to test cobalt (III) because of the tendency of trivalent cobalt to form stable unionized complexes. Several compounds containing trivalent cobalt were investigated ; the compounds used and results

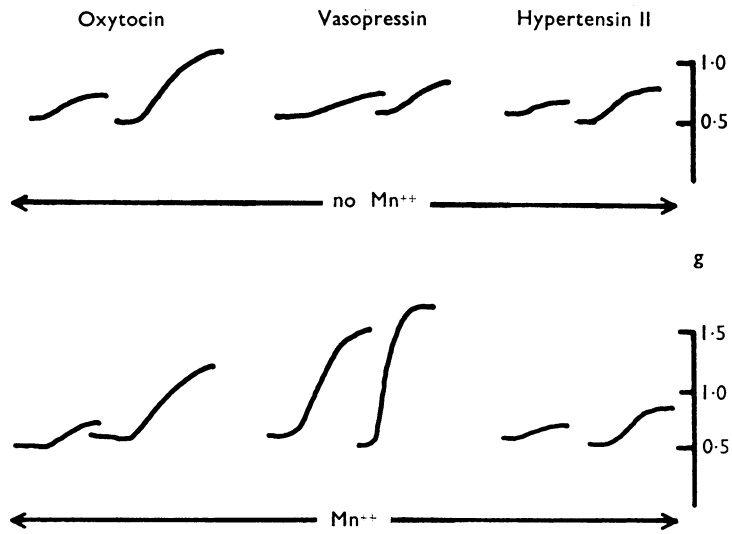

FIG. 10. Effect of $1{\cdot}6 \times 10^{-4}$ M Mn^{++} on responses to oxytocin (1 and 3·3 m-u./ml.) lys-vasopressin (10 and 33 m-u./ml.) and hypertensin II ($1{\cdot}6\times$ and 5×10^{-8} g/ml.). K_2SO_4-Ringer, 1 mM Ca^{++}.

TABLE 4. *Half-times (min) of decay of vasopressin potentiation*

Co^{++}	Mg^{++}
1·7	0·5
1·25	0·5
1·85	0·9
1·0	0·7

obtained are shown in Table 5. A neutral complex, cobaltic acetylacetonate, and a cationic complex, cobaltic hexammine, both failed to produce potentiation (Fig. 11); high doses of the ammine produced some antagonism. The failure of the positively charged ammine complex to potentiate vasopressin is interesting because it shows that a positive charge is insufficient in itself to activate the vasopressin receptor.

In contrast to cationic complexes, an anionic complex, cobaltinitrite, potentiated vasopressin; as shown in Fig. 11 its activity was almost as great as that of an equimolar solution of cobaltous sulphate. Since the cobaltinitrite decomposes in solution with the production of Co^{+++} ions it is possible that this ionic species may have been responsible for the potentiating effect. Alternatively trivalent cobalt may have reverted to the preferred divalent form before exerting its biological action.

The potentiating activity of nickel on the vasopressin receptor is of the same order as that of manganese and cobalt (Table 3). Minor differences in potentiating activity of the three transition metals may not be significant and possibly be connected with differences in their direct action on smooth muscle. Thus manganese normally relaxed the depolarized uterus while nickel contracted it; cobalt tended to produce contraction followed by relaxation.

When response curves for nickel and magnesium were plotted against log metal concentration (as in Fig. 9) parallel lines resulted. The three transition metals tended to give parallel lines with magnesium when plotted in this way; a statistical analysis by the sign test showed no significant deviation from parallelism for the combined results.

Iron

Although iron is placed between manganese and cobalt in the periodic table its potentiating activity for lysine vasopressin was considerably less than that of either of them. Divalent iron ($FeSO_4$) was rather less active than magnesium and trivalent

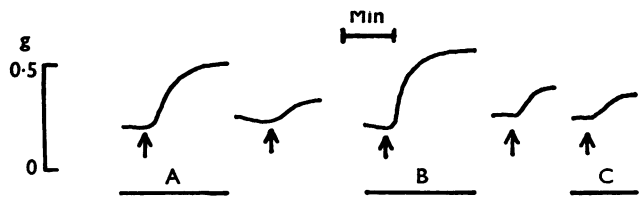

FIG. 11. Effect of cobalt compounds on response to 10 m-u./ml. lys-vasopressin (arrow). A, Sodium cobaltinitrite; B, cobaltous chloride; C, hexammino-cobaltic chloride; all 5×10^{-5} M. K_2SO_4-Ringer, 0·1 mM Ca^{++}.

TABLE 5

Cobaltic salt:	
Cobaltic fluoride, CoF_3	Insoluble
Neutral complex:	
Cobaltic acetylacetonate, $Co(C_5H_7O_2)_3$	Inactive
Cationic complex:	
Cobaltihexammino chloride, $Co(NH_3)_6Cl_3$	Inactive
Anionic complex:	
Sodium cobalticarbonate, $Na_3Co(CO_3)_3$; $3H_2O$	Insoluble
Sodium cobaltinitrite, $Na_3Co(NO_2)_6$	Active

iron ($FeCl_3$) much less active (Table 3). These results must be viewed with caution, however, as divalent iron salts tended to opalesce or precipitate in the organ bath and thus may have lost activity ; trivalent iron is known to be extensively hydrolysed in neutral solution (Gurd *et al.*, 1956) which may account for its lack of activity.

Ferrous sulphate was used on seven occasions ; its activity was generally about one-third that of magnesium, but in some instances it appeared equiactive with it. The dose-response curves with ferrous sulphate were atypical and distinctly steeper than with magnesium, a steep inflexion of the curve occurring in the region 5×10^{-4} to 2×10^{-3}M $FeSO_4$. The potentiating effect of ferrous ion towards vasopressin tended to persist after washout and the application of iron was sometimes followed by persistent impairment of the response. The addition of ferrous salts to the bath usually produced a transient contractile effect.

Copper

Copper (II) sulphate was used in three experiments in 5×10^{-4}M concentration. It failed to potentiate vasopressin, but produced a depression of the response which persisted after washout.

Zinc

Zinc ($ZnSO_4$) caused a distinct potentiation of lysine vasopressin. The activity of zinc was about equal to magnesium but its potentiating effect recovered more slowly ; in some cases the administration of zinc was followed by an impairment of the response to further doses of vasopressin. Zinc failed to potentiate acetylcholine under conditions in which vasopressin was potentiated.

Possible mechanism of metal potentiation

A set of mass-law equations may be formulated based on the assumption that metal ions M can combine with S-S polypeptide receptors R and that S-S polypeptides P can combine either with free receptors R or with metal combined receptors MR to form complexes PR and PMR. As a first approximation it will be assumed that PR and PMR are equally effective in terms of " intrinsic activity " or " efficacy." The following reactions have to be considered

$$P+R=PR$$
$$M+R=MR$$
$$P+MR=PMR$$

The corresponding mass action affinity constants are

$$K_1 = \frac{y_1}{[P]\ (1-y_1-y_2-z)}$$

$$K_2 = \frac{z}{[M]\ (1-y_1-y_2-z)}$$

$$K_3 = \frac{y_2}{[P]\ z}$$

where y_1, y_2 and z represent respectively receptor fractions PR, PMR and MR ; and $[P]$ and $[M]$ are concentrations of polypeptide and metal in the solution. The total activated receptor fraction is

$$y=y_1+y_2$$

At equilibrium

$$y = \frac{K_1[P] + K_3[P]K_2[M]}{1 + K_2[M] + K_1[P] + K_3[P]K_2[M]} \quad \ldots\ldots (1)$$

Some implications of this treatment are as follows:

1. Equation (1) can account for the experimental finding that S-S polypeptides retain activity in the absence of metals, and that the effects of metals on different S-S polypeptides vary. It may be shown that the differential coefficient

$$\frac{dy}{d[M]}$$

contains the factor $[P]$ $(K_3 - K_1)$ in the numerator. Hence increasing metal concentrations would be expected to produce potentiation or depression according to whether for a particular polypeptide the constant K_3 or K_1 is the larger—that is, whether the polypeptide has greater affinity for metal-bound or metal-free receptors.

2. When y in equation (1) is plotted against log $[P]$ for different values of $[M]$, K_1, K_2 and K_3, a series of parallel lines result. This may be shown as follows (see also Schild, 1969). By differentiation of y with respect to log $[P]$

$$\frac{dy}{d \log_{10}[P]} = \frac{dy}{d[P]}[P]2{\cdot}3 = y(1-y)2{\cdot}3$$

Since the derivative is a single-valued function of y, all tangents are identical for a given value of y and hence the curves are parallel.

Previous workers have investigated the slope of log dose-response curves of S-S polypeptides with and without magnesium and concluded that they are parallel (Bentley, 1965 ; Krejci *et al.*, 1968). In the present experiments on depolarized rat uterus, tests for parallelism have given variable results. In a number of experiments in which responses with and without magnesium were recorded in the same effect range, a parallel shift was obtained as shown for example in Fig. 6. Parallelism was the rule in experiments with arginine vasopressin, arginine vasotocin and oxytocin but in some experiments with lysine vasopressin curves flattened in the absence of magnesium. Some preparations became very insensitive towards lysine vasopressin in the absence of magnesium and in such cases the high chlorbutol content of the vasopressin ampoules may have contributed to flattening the slope, but occasionally a distinct decline of slope in the absence of magnesium was observed with moderate doses of lysine vasopressin as shown for example in the experiment illustrated in Fig. 12. The point requires further investigation. A flattening of the

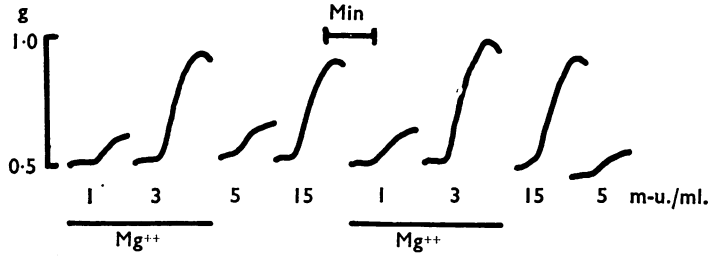

FIG. 12. Lys-vasopressin 1 and 3 m-u./ml. in presence of 1 mM Mg++ and 5 and 15 m-u./ml. in absence of Mg++. Note slightly steeper dose-response with Mg++. K_2SO_4-Ringer, 0·1 mM Ca++.

slope in the absence of metal may mean that the assumption of equal efficacy of PR and PMR will have to be abandoned.

3. The effects of two metals may be expressed, as in Fig 9, by plotting responses (at constant [*P*]) against log [*M*]. If the two metals differed with respect to K_2 parallel curves would be expected (because a change of K_2 in equation (1) with constant product $[M]K_2$ amounts to a parallel shift on the log [*M*] axis). If the two metals differed with respect to K_3 non-parallel curves would be expected. Since magnesium and the three transition metals manganese, cobalt and nickel give parallel curves they may differ in respect of K_2, which expresses the affinity of metal for the receptor.

Empirical approach

The following more empirical approach allows for a quantitative comparison with metal enzymes.

When the velocity of an enzyme reaction is plotted against metal concentration, a curve resembling the Michaelis curve for substrate concentration is often obtained and if these data are treated by the usual graphical methods, an empirical association constant for the metal can be calculated. Such " constants " are known for many metal enzymes—for example, enolase (Warburg & Christian, 1942) and leucine aminopeptidase (Smith & Hill, 1960).

The present data can be treated in a similar way by plotting in a double reciprocal plot (degree of potentiation)$^{-1}$ against (metal concentration)$^{-1}$. An approximately straight line results as shown in Fig. 13 provided that the data are taken from the lower part (below 80%) of the metal response curve and the effect of polypeptide alone is small. Degree of potentiation is here defined as the response in the presence of metal minus response in the absence of metal at constant polypeptide concentration. From the points in Fig. 13, which are means of six determinations, an apparent metal association constant can be derived using the slope and intercept of the line. The empirical association constant for magnesium and lysine vasopressin from Fig. 13 works out at $4{\cdot}4 \times 10^3 \text{M}^{-1}$. The corresponding empirical association constant for magnesium and enolase is $1{\cdot}6 - 2{\cdot}7 \times 10^3 \text{M}^{-1}$ (Warburg *et al.*, 1942) and

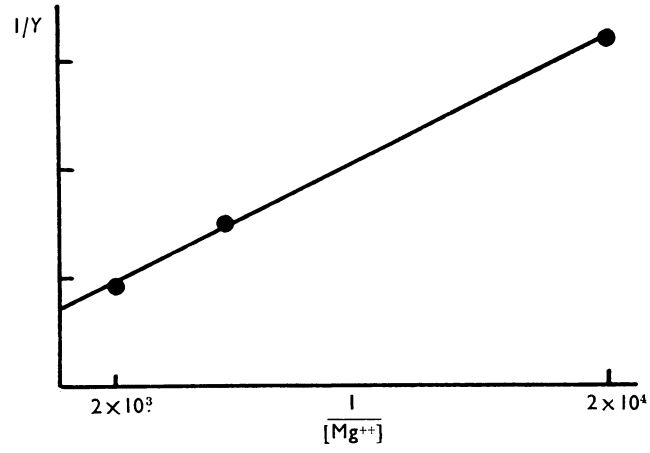

FIG. 13. Plot of $\frac{1}{Y}$ (Y=degree of potentiation as defined in text) against $\frac{1}{[Mg^{++}]}$. Means of six determinations.

for magnesium and leucine aminopeptidase from swine kidney it is $1{\cdot}2 \times 10^4 M^{-1}$ (Smith & Spackman, 1955). Evidently the apparent association constant of magnesium in the S-S polypeptide receptor system is of the same order of magnitude as in enzyme systems.

Discussion

It has been shown that the action of S-S polypeptides in the isolated uterus is potentiated not only by magnesium but by a variety of other metals, some much more active than magnesium. These experiments confirm previous reports that S-S polypeptides are potentiated by manganese and cobalt (Bentley, 1965 ; Somlyo *et al.*, 1966) as well as magnesium. The present findings refer to depolarized muscle, but as previously discussed (Schild, 1967) drug effects in depolarized muscle are likely to be exerted on the same receptors as in normal muscle, while the absence of conducted impulses in this type of preparation simplifies analysis and enables consistent graded responses to be obtained.

That the effect of metals on S-S polypeptides is exerted at the receptor level is suggested by its specificity, other smooth muscle stimulants such as bradykinin, hypertensin and acetylcholine being unaffected by metals under similar experimental conditions. Perhaps no other known drug receptor is as specifically activated by metals as the S-S polypeptide receptor which might thus be referred to as a metal receptor by analogy with the term metal enzyme. Alternatively, the metal itself might be considered as a " co-receptor " by analogy with coenzyme, originally defined by Duclaux in 1897 as " a dialysable substance necessary for enzyme activity " (quoted from Malmström & Rosenberg, 1959).

Comparison with metal enzymes

Metal enzymes may be divided into two classes (Malmström & Rosenberg, 1959 ; Vallee & Coleman, 1964), enzymes with strongly bound metals (metalloenzymes) and enzymes with dissociable metal ions (metal-enzyme complexes). Operationally the former do not require the addition of metal ions to the assay medium while the latter require it to the extent that their activity is determined by the concentration of free metal in solution. The S-S polypeptide receptor shows obvious analogies with the second class both in its dependence on metal concentration in solution and in its quantitative affinity for metals. The apparent association constant of the receptor is 4×10^3 for Mg^{++} and about ten times higher for Mn^{++}, Co^{++} and Ni^{++} which corresponds to typical metal-enzyme complexes.

The effect of metal on the receptor is rapidly reversible, more so than is usually found with metal enzymes, but there is evidence, nevertheless, that the rate of reversal of the potentiating effect of metals is limited by factors other than simple diffusion. Thus it was found that the potentiating effect of cobalt declined at a significantly slower rate than that of magnesium and as their diffusion coefficients (from *International Critical Tables*) are practically identical, this would suggest a difference in their rates of dissociation from receptor. The finding that on changing to a magnesium-free solution the sensitivity of the uterus to vasopressin continued to decline progressively but that it could be restored by adding magnesium is also suggestive of gradual dissociation from a receptor.

Some metal enzymes become irreversibly inactivated in the absence of metals, others are completely inactivated but the inactivation is reversible whilst a third group are incompletely and reversibly inactivated (Vallee & Coleman, 1964). The S-S polypeptide receptor seems to correspond to the third type which can be reversibly but only incompletely inactivated, although it cannot be excluded that a longer and more drastic exposure to a metal free medium might produce irreversible inactivation.

Effects of different metals

Attempts to explain the frequently highly specific effects of metals on enzymes have been generally unsuccessful and only limited generalizations have emerged; some of these, nevertheless, may provide pointers to the possible mode of action of metals on the S-S polypeptide receptor. For example, enzymes mediating oxidation-reduction reactions frequently require metals such as copper and iron which can provide the required redox couple and it may be relevant that these metals were either completely or relatively inactive in the S-S polypeptide system.

The order of activity of different metals in potentiating lysine vasopressin was as follows:

$$Co^{++} \geqslant Co^{+++} \geqslant Mn^{++} \geqslant Ni^{++} \geqslant Mn^{+++} > Zn^{++} \geqslant Mg^{++} > Fe^{++} > Fe^{+++} \geqslant Ca^{++} > Be^{++} = Sr^{++} \leftarrow Ba^{++} = Cu^{++} = O.$$

This sequence represents the crude experimental results and does not necessarily indicate the intrinsic activities of the ions. Thus Co^{+++} and Mn^{+++} may revert to the divalent form; the activity of Cu^{++} may be reduced by hydrolysis and that of Fe^{+++} and Be^{++} almost certainly is so reduced (Gurd *et al.*, 1956). The lack of potentiation by Sr^{++} and Ba^{++} is probably genuine and so is the slight potentiating activity of Ca^{++} which may represent an effect at the receptor level over and above its essential role in the contractile mechanism. Almost certainly genuine is the high activity of the transition metal ions Co^{++}, Mn^{++} and Ni^{++}, and the rather lower activity of Mg^{++}, as these metals do not hydrolyse in the experimental pH range.

TABLE 6. *Empirical crystal radii (Pauling, 1960) and first stability constants K_1 (Sillen & Martell, 1964)*

	Crystal radius	K_1 Ethylenediamine	K_1 Glycine
Co^{++}	0·74	5·9	4·9
Co^{+++}	0·63	(18·7)	
Mn^{++}	0·80	2·7	3·3
Ni^{++}	0·72	7·5	6·0
Mn^{+++}	0·66		
Zn^{++}	0·74	5·7	5·2
Mg^{++}	0·65	0·37	3·0
Fe^{++}	0·76	4·3	4·0
Fe^{+++}	0·64		10·0
Ca^{++}	0·99		1·4
Be^{++}	0·31		
Sr^{++}	1·13		0·8
Ba^{++}	1·35		0·77
Cu^{++}	0·88*	10·7	8·1

* Calculated after Pauling (1960).

Some physico-chemical properties of metals are summarized in Table 6 which should be consulted in conjunction with Table 3 which gives their potentiating activities.

Column 1 shows the ionic crystal radii of metals after Pauling (1960). All the active potentiating metals have crystal radii of roughly similar sizes while Ca^{++} and particularly the inactive Sr^{++} and Ba^{++} have larger radii. (This correlation may however be spurious because it was found in unpublished experiments that Cd^{++} with a crystal radius very close to Ca^{++} has strong though irregular potentiating activity.) Columns 2 and 3 give the first stability constants (K_1) after Sillen & Martell (1964) for a typical nitrogen ligand, ethylenediamine and a typical oxygen-nitrogen ligand, glycine. The metals are listed in the order of their potentiating activity. There is obviously no simple correlation between the potentiating activities of different metals and their stability constants except that metals with very little coordinating activity such as Ca^{++} and those with very high coordinating activity such as Cu^{++} have little or no potentiating activity. Klotz (1954) has suggested that metals which form complexes with great avidity may not be good enzyme activators because they have no open coordination positions all of which are occupied by OH^- ions at pH above 7. In this context the high potentiating activity of cobaltinitrite seems surprising unless it is assumed that the active moiety is not the strongly complexing Co^{+++} but Co^{++}.

Mechanism of action

In principle the metal could react with the receptor or with the polypeptide or with both forming a bridge between them. The possibility that the metal reacts primarily with the polypeptide in solution cannot be entirely excluded but is rendered less likely by the evidence, which has been referred to, of an interaction between metal and receptor. Only a few of several possible mechanisms will be discussed (see also Bentley, 1965).

1. The metal influences the conformation of the receptor without direct interaction with it, possibly by an allosteric effect. This is a feasible mechanism.

2. The metal itself activates the receptor, say by charge neutralization. This attractive hypothesis fails to explain the high activity of some S-S polypeptides in the absence of metal.

The following mechanisms assume a bridging function of the metal. That metals may form ternary complexes with proteins and small molecules has been demonstrated by Klotz & Ming (1954) and repeatedly confirmed.

3. The metal provides a link between receptor and certain reactive chemical groups present only in S-S polypeptides which are potentiated by metals. Although such a scheme would be compatible with the kinetic mechanism discussed, it is difficult to reconcile with some of the experimental data on the effect of metals on S-S polypeptides. Thus the different effect of metals on oxytocin and lysine vasopressin might be explained by metal coordination with the ε-amino group of lysine but the large differences in metal potentiation between oxytocin and val_3-oxytocin found by Bentley (1965) and Munsick & Jeronimus (1965) could not be explained as the two compounds differ only by an unreactive methyl group.

4. The polypeptide attaches to the receptor by its normal attachment groups but in addition the metal forms a link between receptor and polypeptide which aids

their apposition and interaction. A somewhat similar mechanism has been proposed for certain metal enzymes (Klotz, 1954; Mildvan & Cohn, 1966). This type of mechanism might explain the finding (brought out by comparing Table 5 of Munsick, 1968 and Tables 2 etc. of Berde and Boissonnas, 1968) that polypeptides which are less active on the uterus than oxytocin are more potentiated than oxytocin by magnesium; presumably their apposition to the receptor is deficient and can be improved by magnesium. Conversely desaminooxytocin which is definitely more active than oxytocin is if anything less potentiated by magnesium than oxytocin.

It is implicit in this hypothesis that metals can coordinate with S-S polypeptides. This seems reasonable in view of the many potential electron donor groups in these molecules, not only the α-amino and other side groups, but presumably also the S-S group and the oxygen and nitrogen in the amide linkages.

A crude picture might be that metals of a certain ionic radius can fit into the receptor and attract the S-S polypeptide molecule; the weak potentiating action of magnesium could be explained by its relatively weak coordinating power with ligands in the receptor surface (perhaps also with ligands in the polypeptide molecule) but other aspects of metal specificity remain unexplained, for example the similar activity of manganese and cobalt in spite of marked differences in their ligand affinities (Table 6).

The above mechanism would be compatible with the kinetic scheme which has been discussed, but in general the present evidence from dose-response curves is insufficient to decide between mechanisms. It is indeed doubtful whether such evidence can ever be decisive although it may provide useful pointers. Kinetic evidence alone can seldom be decisive in enzymology and the argument applies with even greater force to pharmacological dose-response curves. A variety of approaches based on different methods will be needed to clarify further the mechanism of metal potentiation of the S-S polypeptide receptor. An important gap in our present knowledge is the lack of quantitative data on the stability constants of S-S polypeptide ligands and metal ions.

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