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THE EFFECT OF COPPER SULPHATE AND MANGANESE SULPHATE ON THE TOXICITY AND TREMOR OF TREMORINE AND ON SOME PERIPHERAL RESPONSES INDUCED BY ACETYLCHOLINE, NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

P. M. CEASAR and H. SCHNIEDEN

Department of Pharmacology, University of Manchester

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Abstract—Copper sulphate and manganese sulphate markedly potentiated the toxicity of tremorine in mice. Neither metal salt caused tremor on its own. Copper sulphate, administered prior to tremorine, increases the tremor and appears to prolong it. Manganese sulphate caused a significant increase in tremor only in the early stages of the experiment.

Copper sulphate, at a dose of 20 μg/ml significantly potentiated the response of guinea pig ileum to acetylcholine, whereas manganese sulphate inhibited the standard response at all dose levels. On the same preparation, both metal salts inhibited the responses to a standard dose of 5-hydroxytryptamine. All doses of copper sulphate used inhibited the noradrenaline standard response of guinea pig vas deferens and also caused inhibition of the response to dopamine at the 2 and 20 μg/ml dose levels of metal salt. However, at a high dose of 200 μg/ml copper sulphate there was non-significant potentiation of the dopamine response. Manganese sulphate, in doses of 2 and 20 μg/ml potentiated responses to both catecholamines, but markedly inhibited responses at high doses of metal salt.

CURZON AND SCHNIEDEN[1] showed that copper sulphate potentiated both the toxicity and tremor of NN-diethylcysteamine. Tremorine (1, 4-dipyrrolidino-2-butyne) can also cause tremor.[2] In Wilson's disease and Manganism excess amounts of copper and manganese occur in the body and are associated with extrapyramidal disorders in which tremor is a common feature. In view of this, the effect of copper sulphate and manganese sulphate on the toxicity and tremor caused by tremorine in mice has been investigated.

Tremorine is also known to increase the concentration of brain acetylcholine,[3, 4] and to affect brain monoamine levels.[5, 6] It was therefore of interest to examine the effects of a copper and manganese salt on the responses of some isolated tissues to acetylcholine, noradrenaline, dopamine and 5-hydroxytryptamine.

METHODS

Male albino mice (T T strain), weighing between 17 and 30 g, were used for the determination of the effects of copper sulphate and manganese sulphate on tremorine toxicity and tremor.

* Present address: Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, Goldhawk Road, London, W.6.

Acute toxicity determination

The mice were placed singly in glass jars. Experiments were conducted in a constant temperature room at 25·5° ± 1°. Injections were given by the intraperitoneal route and the animals were allowed free access to food and water up to the time of the injection. One hour LD_{50} values were determined for copper sulphate, manganese sulphate and tremorine. The effect of a dose of copper sulphate and manganese sulphate, below the theoretical LD_0 of these drugs, on the LD_{50} value of tremorine was then determined. A control group of mice were injected with 0·25 ml of 0·9% sodium chloride solution (saline). The LD_{16}, LD_{50} and LD_{84}, and estimate of relative potency, were calculated by the method of Litchfield and Wilcoxon.[7]

Tremor studies

The tremor recording apparatus was a modification of that described by Ahmed and Taylor.[8] The output from the gramophone pick-up was fed to a frequency selective amplifier, the output of which charged a capacitor. The amount of movement of a mouse registered as a reading on an arbitrary scale, between 0 and 50, on a micro-ammeter. The amplifier was most sensitive to frequencies of about 11 c/s and thus amplified frequencies of the order of those found in tremor to a greater extent than those of ordinary walking movements. Experiments were performed at a temperature of 25·5° ± 1°. A mouse was placed in each of four plastic containers which were then suspended from the needle-hole of a gramophone pick-up. The animals were allowed to settle down for about 30 min, after which they were treated with test drug or control. All injections to the mice were given by the intraperitoneal route. The mice were injected with either 0·9% saline as control, tremorine alone, copper sulphate plus tremorine or manganese sulphate plus tremorine. The first tremor recording was taken 10 min after injection of tremorine and thereafter every 5 min over a period of 1 hr. Where a metal salt was given, it was injected 15 min before the tremorine. The tremor in a mouse occurred in short bursts. Therefore, to obtain a satisfactory recording of the tremor, six readings were taken over a period of 1 min. Each reading lasted 5 sec e.g., for a recording of tremor at the 10th minute after injection of tremorine, readings were taken from 0 to 5 sec, 10 to 15 sec, 20 to 25 sec, 30 to 35 sec, 40 to 45 sec and 50 to 55 sec. The mean of these six readings during the 10th minute was taken as the tremor score for that minute.

Isolated tissues

Acetylcholine and 5-hydroxytryptamine. Guinea pig ileum. Guinea pigs, of either sex and weighing 300–600 g were used. A length of gut of about 4 cm was removed from the distal end of the ileum. This was immersed in Tyrode solution contained in a 20 ml organ bath at a temperature of 33·5° ± 0·5°. The solution was aerated by a mixture of 95% oxygen and 5% carbon dioxide. Contractions of the gut were recorded with an isotonic frontal lever on a smoked paper kymograph. Magnification of the response was about six times. Loads of 0·5–1·0 g and 0·5 g were placed at the point of attachment of thread to the lever for the experiments with acetylcholine and 5-hydroxytryptamine respectively.

For both sets of experiments, standard doses of either acetylcholine or 5-hydroxy-tryptamine were added to the bath at 5-min intervals. The method used was to obtain three standard contractions of the ileum about 50 per cent of the maximum, to either

acetylcholine or 5-hydroxytryptamine. The former was added to the bath for a contact time of 30 sec and the latter for a contact time of 25 sec. In the next cycle, copper sulphate or manganese sulphate was added 30 sec before the drug. The drug was then added to the bath for a contact time similar to that used during the control period. The preparation was washed several times, at constant intervals, after addition of acetylcholine and 5-hydroxytryptamine.

Noradrenaline and dopamine. Guinea pig vas deferens. The isolated guinea pig vas deferens was prepared as described by Leach.[9] Male guinea pigs of 300–600 g were used. Conditions were similar to those described for the ileum experiments but a temperature of 31·5 ± 0·5° was used. For noradrenaline experiments the response was magnified about seven times with a 0·5-g load on the tissue at the point of attachment to the lever. Corresponding figures for dopamine were eight times magnification and 1-g load.

Five minutes was found to be a satisfactory cycle time for the noradrenaline experiments, with a drug contact time of 1 min. Three standard submaximal contractions were established for the noradrenaline standard dose. Then the effect of either copper sulphate or manganese sulphate on the standard noradrenaline response was examined, the metal salt being added 30 sec before the catecholamine. In all experiments the tissue was washed several times at constant intervals following addition of drugs to the bath.

The dopamine experiments were conducted in a similar manner to those involving noradrenaline, except that a 7-min cycle was used, and a contact time of 2 min, 15 sec was required owing to a lag period before the appearance of the dopamine response.

Drugs used

Copper sulphate ($CuSO_4,5H_2O$), Analar grade. Manganese sulphate ($MnSO_4, 4H_2O$). Analar grade. Tremorine dihydrochloride* (1,4-dipyrrolidino-2-butyne dihydrochloride). L-Noradrenaline bitartrate.* Serotonin creatinine sulphate.* Acetylcholine chloride* and 3-hydroxytyramine hydrochloride.*

In the text above, items marked with an asterisk* will be referred to as tremorine, noradrenaline, 5-hydroxytryptamine, acetylcholine and dopamine respectively. All concentrations of drugs are expressed in terms of drug *salt* not base e.g., 1 μg noradrenaline refers to 1 μg L-noradrenaline bitartrate. Copper sulphate and manganese sulphate refers to their respective hydrated salts.

RESULTS

The 1 hr i.p. toxicities of tremorine, copper sulphate and manganese sulphate in the mouse are shown in Table 1. No mice died in the control group of mice injected with normal saline.

The theoretical maximum LD_0 of copper sulphate was calculated to be 39 mg/kg body wt. and that of manganese sulphate to be 370 mg/kg body wt. Doses of metal salt below these calculated LD_0's i.e. 30 mg/kg copper sulphate or 300 mg/kg manganese sulphate were injected into mice 25 min before injecting the same animals with the LD_{50} dose of tremorine, i.e. 315 mg per kg body wt.

From Table 2 it can be seen that administration of copper sulphate potentiates the toxicity of tremorine, increasing the percentage death from 50 to 100 per cent. Manganese sulphate also has a marked potentiating effect on the toxicity of tremorine.

The effects of copper sulphate (30 mg/kg) and manganese sulphate (100 mg/kg) on tremorine tremor in mice is shown in Table 3. The metal salt was injected 15 min

TABLE 1. THE 1-HR TOXICITIES OF INTRAPERITONEAL TREMORINE, COPPER SULPHATE AND MANGANESE SULPHATE IN THE MOUSE

Drug	No. of animals used	LD_{16}	LD_{50}	LD_{84}
Tremorine	106	255	315 (296–335)	389
Copper sulphate	90	51	89 (75–106)	155
Manganese sulphate	54	414·5	534 (479–595)	684

The doses are given in terms of mg/kg body wt. The figures in brackets represent the 95 per cent confidence limits for the LD_{50}.

TABLE 2. THE EFFECT OF COPPER SULPHATE AND MANGANESE SULPHATE ON THE 1-HR LD_{50} OF TREMORINE

Treatment	Dose	No. of animals used	No. dead after tremorine	% Death
0·9% saline; tremorine 25 min later	0·25 ml 315 mg/kg	16	8	50
Copper sulphate; tremorine 25 min later	30 mg/kg 315 mg/kg	16	16	100
Manganese sulphate; tremorine 25 min later	300 mg/kg 315 mg/kg	16	15	93·75
Copper sulphate alone	30 mg/kg	6	0	0
Manganese sulphate alone	300 mg/kg	6	0	0

prior to tremorine. Neither salt caused tremor on its own nor appeared to interfere with the characteristic autonomic effects of tremorine. Tremor could not be evaluated when higher doses of manganese sulphate were used (i.e., 300 or 200 mg/kg) as the combination of these doses with the tremorine proved too toxic to the mice.

Isolated tissue experiments (*see Tables* 4 *and* 5 *and Figs.* 1 *and* 2)

Copper sulphate, in a dose of 20 μg/ml produced some potentiation of the response of the ileum to a standard dose of 0·02 μg/ml acetylcholine, but, at 2 μg/ml, it had no significant effect. Following the addition of 200 μg/ml copper sulphate to the bath potentiation occurred but the baseline did not return to the control position after washing.

TABLE 3. THE EFFECT OF A PREVIOUS INJECTION OF COPPER SULPHATE OR MANGANESE SULPHATE ON TREMORINE TREMOR

Treatment No.		Mean tremor score at times after tremorine or saline			
		10 min	15 min	20 min	40 min
Tremorine (30 mg/kg) (1)		28·30	22·49	20·43	11·33
Copper sulphate (30 mg/kg) 15 min later tremorine (30 mg/kg) (2)		29·45	30·96	29·03	17·98
0·25 ml 0·9% saline (3)		11·43	9·80	9·98	5·42
Tremorine (30 mg/kg) (4)		19·52	14·89	17·67	6·87
Manganese sulphate (100 mg/kg) 15 min later tremorine (30 mg/kg) (5)		26·10	21·30	21·53	15·08
0·25 ml 0·9% saline (6)		6·63	7·80	9·08	7·75
Mann–Whitney Test[10]	comparing (1) and (2)	N. S.	*	*	*
	comparing (1) and (3)	†	†	*	N. S.
	comparing (4) and (5)	*	N. S.	N. S.	†
	comparing (4) and (6)	†	*	*	*

* Significant at 5 per cent level, † at 1 per cent level. N.S.—not significant. Each reading is the mean of ten experiments.

TABLE 4. THE EFFECT OF COPPER SULPHATE ON STANDARD SUBMAXIMAL CONTRACTIONS INDUCED BY ACETYLCHOLINE (0·02 μg/ml) AND 5-HYDROXYTRYPTAMINE (0·2 μg per ml) ON ISOLATED GUINEA PIG ILEUM AND BY NORADRENALINE (2 μg/ml) AND DOPAMINE (5 μg/ml) ON ISOLATED GUINEA PIG VAS DEFERENS

	Concentration of copper sulphate (μg/ml)	No. of animals used	Mean % increase (+) or decrease (−) in response ± S.E. produced by metallic salt	*P* (Mann-Whitney[10] Test)
Acetylcholine	2	12	(−) 3·91 ± 4·30	N.S.
	20	12	(+)31·94 ± 6·33	< ·0014
	200	10	(+)14·27 ± 6·84	< ·0054
5-Hydroxy-tryptamine	2	6	(−)14·26 ± 4·32	< ·002
	20	7	(−)29·36 ± 5·83	< ·00014
	200	5	(−)49·78 ± 9·32	< ·002
Noradrenaline	2	6	(−)24·35 ± 5·65	< ·002
	20	5	(−)64·44 ± 10·49	< ·002
	200	8	(−)71·19 ± 5·94	< ·0003
Dopamine	2	5	(−)19·54 ± 8·52	= ·02
	20	6	(−)40·78 ± 12·31	< ·02
	200	5	(+)37·85 ± 14·32	> ·05 N.S.

N.S.—Not significant.

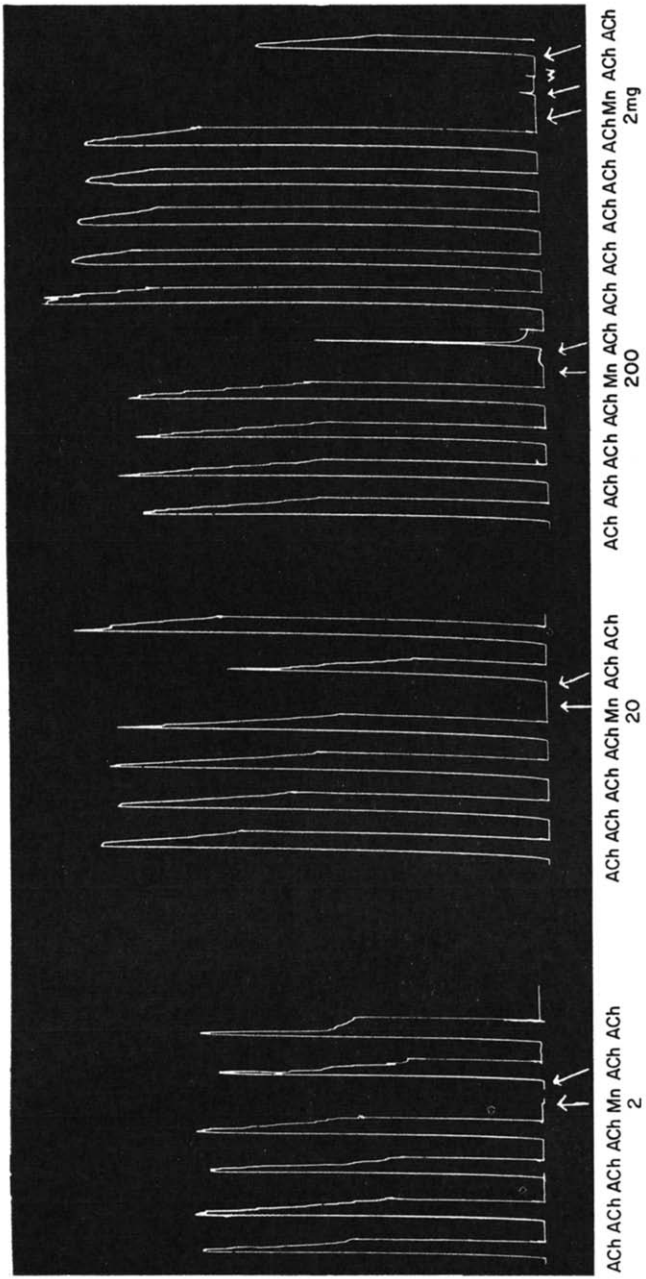

FIG. 1. Guinea pig ileum: The effect of manganese sulphate at four dose levels, on standard contractions induced by 0·4 μg/ml acetylcholine. Manganese sulphate added 30 sec before acetylcholine
ACh—acetylcholine; Mn—manganese sulphate (doses in μg/ml or in mg/ml where shown).

TABLE 5. THE EFFECT OF MANGANESE SULPHATE ON STANDARD SUBMAXIMAL CONTRACTIONS INDUCED BY ACETYLCHOLINE (0·4 μg/ml) AND 5-HYDROXYTRYPTAMINE (0·2 μg/ml) ON ISOLATED GUINEA PIG ILEUM AND BY NORADRENALINE (1 μg/ml) AND DOPAMINE 5 μg/ml) ON ISOLATED GUINEA PIG VAS DEFERENS

	Concentration of manganese sulphate (μg/ml)	No. of animals used	Mean % increase (+) or decrease (−) in response ± S.E. produced by metallic salt	*P* (Mann–Whitney[10] Test)
Acetylcholine	2 μg/ml	5	(−) 9·64 ± 3·35	< ·05
	20 μg/ml	5	(−)22·45 ± 3·53	< ·002
	200 μg/ml	5	(−)59·62 ± 5·27	< ·002
	2 mg/ml	5	(−)96·27 ± 0·53	< ·002
5-Hydroxy-tryptamine	2 μg/ml	5	(−)18·35 ± 5·89	< ·002
	20 μg/ml	5	(−)46·88 ± 5·08	< ·002
	200 μg/ml	4	(−)94·83 ± 1·77	< ·002
	2 mg/ml	5	(−)100	< ·002
Noradrenaline	2 μg/ml	8	(+)20·84 ± 7·24	< ·006
	20 μg/ml	9	(+)24·96 ± 27·39	N.S. > 0·3
	200 μg/ml	5	(−)92·49 ± 3·28	< ·002
	2 mg/ml	6	(−)95·62 ± 0·71	< ·002
Dopamine	2 μg/ml	8	(+) 7·94 ± 8·21	N.S. > 0·7
	20 μg/ml	9	(+)38·57 ± 9·84	< ·00032
	200 μg/ml	9	(−)95·42 ± 4·13	< ·002
	2 mg/ml	5	(−)100	

N.S.—Not significant.

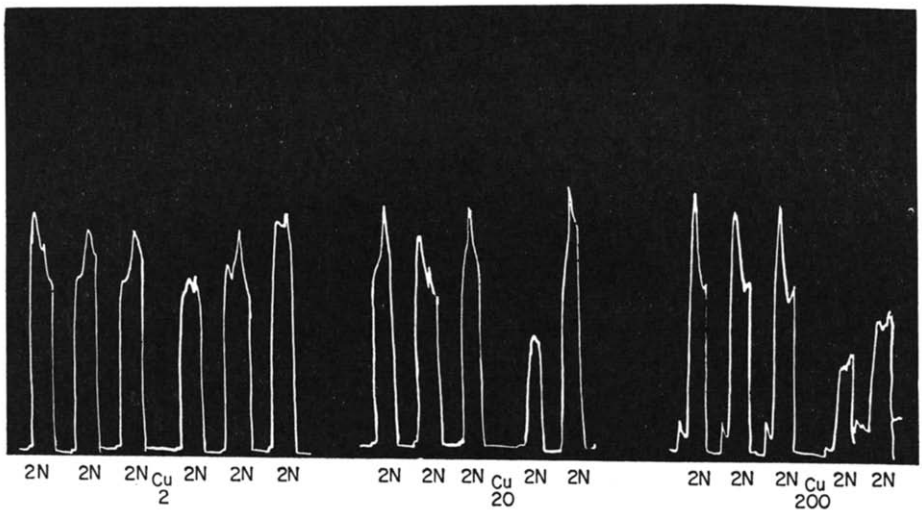

FIG. 2. Guinea pig vas deferens: The effect of copper sulphate at three dose levels, on standard contractions induced by 2 μg/ml noradrenaline. Copper sulphate added 30 sec before noradrenaline. N—noradrenaline; Cu—copper sulphate (μg/ml).

Inhibition of the responses to a standard dose of 0·4 μg/ml acetylcholine was observed at all dose levels of manganese sulphate used. The standard response to acetylcholine could not be restored following the addition of 2 mg/ml manganese sulphate to the bath.

The addition of 200 μg/ml copper sulphate and 2 mg/ml manganese sulphate to the bath always resulted in the appearance of a white precipitate in the Tyrode solution. This precipitate was revealed by chemical analysis to be due to precipitation of sparingly soluble metal phosphate. Only a small portion of the total amount of metal ion was involved in phosphate formation. Copper sulphate at dose levels of 2, 20 and 200 μg/ml inhibited the response of the ileum to a standard 0·2 μg/ml dose of 5-hydroxytryptamine. Manganese sulphate, at all the dose levels used, also inhibited the response of the ileum to the standard 0·2 μg/ml dose of 5-hydroxytryptamine. After addition of 2 mg/ml manganese sulphate to the bath the muscle would not respond to a standard dose of 5-hydroxytryptamine on washing. More washing than normal was required to bring the lever back to the baseline following addition of 200 μg/ml copper sulphate than at lower dose levels. The response to the standard dose of 5-hydroxytryptamine was then restored to normal.

Copper sulphate and manganese sulphate did not cause any contraction of the vas deferens in the 30-sec period prior to addition of noradrenaline or dopamine. Copper sulphate inhibited the response of the tissue to a standard 2 μg/ml dose of noradrenaline. This occurred at all the dose levels of copper sulphate used i.e., 2, 20 and 200 μg/ml. The response to the standard dose of noradrenaline after washing was found to be about 50 per cent of that prior to addition of 200 μg/ml copper sulphate.

A dose of 2 μg/ml manganese sulphate potentiated the response to the standard 1 μg/ml dose of noradrenaline. At the 20 μg/ml dose level the effect of manganese sulphate on the noradrenaline response was very variable, ranging from marked inhibition to marked potentiation. Very marked inhibition of the noradrenaline response occurred at the dose levels of 200 μg/ml and 2 mg/ml manganese sulphate. Following these doses the response to a standard dose of noradrenaline could not be restored.

When dopamine was added to the organ bath, a lag period of about 45 sec occurred before a contraction was observed. Inhibition of the standard response to 5 μg/ml dopamine occurred at the dose levels 2 and 20 μg/ml copper sulphate but some potentiation at the dose level of 200 μg/ml copper sulphate. At the latter dose a red coloration developed in the bath fluid on addition of dopamine, and several additional washes were required to bring the baseline back to its control position.

Manganese sulphate, at the dose levels of 2 and 20/μg/ml, potentiated the response to the standard 5/μg/ml dose of dopamine. Marked inhibition was caused by addition of 200/μg/ml and 2 mg/ml of manganese sulphate and a red coloration developed in the bath fluid when dopamine was added to the bath in the presence of 200/μg/ml manganese sulphate. The inhibitory effect of 2 mg/ml manganese sulphate was not reversible with washing.

DISCUSSION

The acute toxicity of tremorine was potentiated by both manganese sulphate and copper sulphate. Bienvenu *et al.*,[11] found that the sulphate radical has only a weak effect in the determination of the i.p. toxicities of these metal salts in mice, so the increase in toxicity is probably associated with the metal ions.

The means whereby such ions could potentiate the toxicity and the tremorigenic effect of tremor is a matter for speculation. Two possibilities are inhibition of enzymes by the metals or chelation of the metals with aromatic amines.[12, 13] Though both possibilities are feasible there is evidence that changes in aromatic amine levels may be associated with tremor[6, 14, 15] and therefore chelation of amines with the metal appears the more likely explanation. Some support for this possibility is offered by the fact that some of the biologically important divalent metal ions frequently exhibit the following order of stability: Cu^{++}, Co^{++}, Fe^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Ca^{++}.[16] One might therefore expect copper to form more stable complexes than manganese with biogenic amines and hence have a longer duration of action. It is of interest that in fact tremorine tremor was potentiated longer by the copper than by the manganese salt.

A potentiation of acetylcholine on the guinea pig ileum could be produced by certain concentrations of copper. This agrees with the findings of Mercier[17] and Godfraind and Godfraind De Becker.[18, 19] The latter workers also noted that the potentiation could occur following a short contact time between the metallic salt and the tissue.

The inhibition caused by manganese sulphate is in agreement with the results of Delaveau,[20] on the isolated intestine of the rat, the guinea pig and rabbit. He obtained similar results with the chloride and the sulphate, and Bohr,[21] has stated that substitution of sulphate or phosphate for chloride has little effect on the contractile response of smooth muscle. Hence it is likely that the metallic cations are important in the responses reported.

High concentrations of both metallic salts produced a red coloration with dopamine. However, it is unlikely that the cause of this coloration was responsible for the marked potentiation with copper sulphate since the opposite effect was produced in the vas deferens by manganese sulphate in the presence of dopamine. The potentiation of catecholamine response observed at the 2 and 20 μg per ml manganese sulphate levels is difficult to explain but for some enzyme systems *in vitro* manganese seems to be able to replace magnesium as an essential metal.[22] Magnesium, itself, has both an enhancing and depressing action on smooth muscle.[21] The effects of manganese ions *in vitro* might be of a similar nature.

On the ileum both copper sulphate and manganese sulphate inhibited the response to 5 hydroxytryptamine at all dose levels. As mentioned previously chelation may be implicated in these phenomena.

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