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THE EFFECT OF COPPER SULPHATE ON THE LD_{50} OF CYSTEAMINE AND NN-DIETHYLCYSTEAMINE AND ON TREMOR INDUCED BY THESE COMPOUNDS

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Abstract—The toxicity of NN-diethylcysteamine but not of cysteamine towards mice was markedly potentiated by copper sulphate. Both of the above sulphydryl compounds when given in sub-lethal doses caused tremor in mice which was more apparent during movement. The tremor caused by NN-diethylcysteamine was potentiated by copper sulphate.

ALTHOUGH many drugs are known which cause static tremor (tremorine, phenothiazines, reserpine etc.), drug-induced intention tremor has been little investigated. Koch & Hagen[1] considered that a number of N substituted derivatives of cysteamine and of cysteamine homologues caused intention tremor when injected subcutaneously into mice. Stern, Ratkovic & Fuks[2] investigated the effects of centrally acting drugs on the tremor caused by NN-diethylcysteamine and showed that in some cases potentiation occurred. They also found that if 10 mg/kg body weight of copper sulphate were injected intraperitoneally 15 min after a NN-diethylcysteamine injection of 50 mg/kg then 5/5 mice died while none of the mice which were only injected with NN-diethylcysteamine died. This observation of an interaction between copper and a possible intention tremor producing agent is of interest in relation to Wilson's Disease in which defective copper metabolism leads to elevated brain copper[3] and in which intention tremor frequently occurs.[4] The effect of copper upon the tremor produced by cysteamine and NN-diethylcysteamine has therefore been investigated. Also in view of the observation of Stern *et al.*[2] that copper potentiates the toxicity of NN-diethylcysteamine more detailed studies were made of the effect of copper upon the toxicities of this substance and cysteamine.

METHODS

Chemicals

The following substances were used: cysteamine hydrochloride (California Biochemicals Inc.), NN-diethylcysteamine (Degussa, Frankfurt. a.M., Germany) and A.R. copper sulphate pentahydrate. The cysteamine was stored over calcium chloride in a dessicator. The NN-diethylcysteamine was received in a sealed ampoule from which it was removed and subdivided while passing nitrogen into 0·5 ml amounts which were then sealed into small ampoules. Before each experiment, samples of the sulphydryl compounds were brought to pH 7·2 $\pm$ 0·2 by the addition of 2·5N sodium

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hydroxide or 2N hydrochloric acid. To prevent oxidation of these solutions, nitrogen was bubbled slowly through them until they were injected. Samples were taken for analysis in terms of sulphydryl content by the method of Alexander.[5] Some comment upon the validity of this procedure is necessary. An analytical method was used in preference to simply weighing the drugs because of the great hygroscopicity of cysteamine hydrochloride and because of the ease of oxidation of both sulphydryl compounds to disulphides. The assay method gave values up to 10% lower than those obtained by weighing. Insofar as this difference is due to the presence of the disulphide compounds it is a source of ambiguity. On one hand, it was found in agreement with Koch and Hagen,[1] that the toxicity of both oxidised and unoxidised cysteamine preparations were comparable. On the other hand, disulphide compounds are not able to complex with copper in the same way as sulphydryl compounds.

Animals

Male mice of I.C.I. or Porton strain of weights ranging between 19 and 33 g were used.

Determination of toxicity

For the determination of LD_{50} the animals were housed singly in glass jars in a constant temperature room at 25° ± 1°. All animals were allowed food and water *ad lib.* up to the time of injections which were given intraperitoneally. When investigating the effect of copper sulphate upon the LD_{50} of cysteamine or NN-diethylcysteamine, a control group of mice injected with the sulphydryl compounds and water were injected at the same time. The LD_{16}, LD_{50}, LD_{84} and estimate of potency where necessary were calculated by the method of Lichfield and Wilcoxon.[6]

Tremor studies

Preliminary trials were done to determine the doses of the sulphydryl compounds which given alone caused a just discernible tremor. The animals were then injected in pairs intraperitoneally, one animal receiving the sulphydryl compound and copper, the other the sulphydryl compound and water. At suitable times the animals were placed on a wire mesh platform ($\frac{3}{4}$ in. mesh) and observed for approximately 1 min. Tremor was recorded by two observers using an arbitary ranking scale of 0–3 (0, no tremor–3, gross tremor). Between observations, the animals were housed singly in glass jars in the constant temperature room as described above. A double blind procedure was used so that neither observer knew which solution was the control and which was the copper containing solution. The dilution of the copper containing solution was such that its identity was not detectable by its colour. The Sign test[7] was used to judge if copper significantly modified the tremor response of the cysteamine or NN-diethylcysteamine treated animals.

RESULTS

Table 1 gives the intraperitoneal toxicities of cysteamine, NN-diethylcysteamine and copper sulphate in the mouse. From this, the theoretical maximum LD_0 of copper sulphate was calculated to be 8·9 mg $Cu\,SO_4.\,5H_2O$/kg body wt. The effect of copper sulphate upon the toxicity of cysteamine was only of borderline significance (see Table 2). It was noted that turbidity occurred when the above two compounds were mixed in the syringe immediately before injection. In contrast, copper sulphate solution

mixed with NN-diethylcysteamine remained clear. The toxicity of NN-diethylcysteamine was markedly potentiated by copper sulphate whether the latter was injected 15 min before, together with or 15 min after the sulphydryl compound. All the data in Table 2 passed the test for homogeneity of Litchfield and Wilcoxon.[6]

TABLE 1. THE TOXICITIES OF INTRAPERITONEAL CYSTEAMINE, NN-DIETHYLCYSTEAMINE AND COPPER SULPHATE IN THE MOUSE

Drug	Number of animals used	LD_{16}	LD_{50} mg./kg body wt	LD_{84}
Cysteamine	70	282	305 (206–314)	331
NN-diethyl-cysteamine	58	76	96 (87–107)	125
$CuSO_4$ $5H_2O$	50	25	33 (27–41)	41

Doses of the sulphydryl compounds are in terms of the free bases. Figures in brackets give the Confidence Limits (P = 0·95) of each LD_{50}.

TABLE 2. THE EFFECT OF COPPER SULPHATE ON THE LD_{50}, LD_{16} AND LD_{84} OF CYSTEAMINE AND NN-DIETHYLCYSTEAMINE

Treatment	Number of animals used	LD_{16}	LD_{50} mg/kg body wt.	LD_{84}	Relative Potency
Water cysteamine; 15 min later	34	270	310 (297–324)	343	1·1 (1·0–1·2)
Copper sulphate; cysteamine 15 min later	35	261	288 (274–302)	319	P = 0·05
Water + cysteamine together	34	284	311 (293–329)	340	0·9 (0·8–1·0)
Copper sulphate + cysteamine together	35	315	341 (297–361)	370	P = 0·05
Water; NN-diethylcysteamine 15 min later	40	83·6	91·8 (88·2–95·5)	101·6	2·3 (2·0–2·7)
Copper sulphate; NN-diethylcysteamine 15 min later	30	32·1	39·8 (33·7–47·0)	49·2	P < 0·05
Water + NN-diethylcysteamine together	29	84·3	90·8 (86·9–94·9)	96·8	2·3 (2·0–2·6
Copper sulphate + NN-diethylcysteamine	21	34·0	40·3 (35·0–46·3)	47·9	P < 0·05
NN-diethylcysteamine; water 15 min later	26	86·7	93·5 (87·4–100·0)	100·9	1·4 (1·1–1·7)
NN-diethylcysteamine; copper sulphate	25	49·2	69·2 (55·3–86·5)	97·7	P < 0·05

All drugs were injected intraperitoneally. Doses of the sulphydryl compounds are in terms of the free bases. 7·9 mg Cu $SO_4.5H_2O$/kg. body weight was injected as a 0·79 mg/ml solution. 10 ml water/kg body weight was injected. Figures in brackets give Confidence limits (P = 0·95). Relative potency is defined as LD_{50} (control)/LD_{50} (copper complex)

The tremor produced by both sulphydryl compounds was much more apparent when the animals moved and thus bears some similarity to intention tremor in man. It can be seen from Table 3 that copper sulphate injected 15 min before NN-diethylcysteamine significantly increased tremor. Results are given in greater detail in Table 4. Some of the animals which were injected with copper sulphate died a few minutes after tremor was recorded. This was not observed with the controls. No potentiation

TABLE 3. THE EFFECT OF COPPER SULPHATE ON THE TREMOR PRODUCED BY CYSTEAMINE AND NN-DIETHYLCYSTEAMINE

Treatment	Number of animals used	T min	Significance of Difference from controls
Water: cysteamine 15 min later	12	3, 7, 12, 30, 60	Not significantly different
Copper sulphate; cysteamine 15 min later	12		
Cysteamine; water 15 min later	6	3, 7, 12, 30, 60	Not significantly different
Cysteamine; copper sulphate 15 min later	6		
Water; NN-diethylcysteamine 15 min later	20	5	Significant $P < 0.01$
Copper sulphate; NN-diethylcysteamine 15 min later	20		
NN-diethylcysteamine; water 15 min later	10	5, 8	Not significantly different
NN-diethylcysteamine; copper sulphate 15 min later.	10		

All drugs were injected intraperitoneally. 129 mg/kg body weight of cysteamine and 48·5 mg/kg body weight of NN-diethylcysteamine were injected (doses in terms of the free bases). 7·9 mg $CuSO_4.5H_2O$/kg. body weight was injected as a 0·79 mg/ml solution 10 ml water/kg body weight was injected. T = the times after the second injection at which tremor was recorded.

TABLE 4. THE EFFECT OF PREVIOUS INJECTION OF COPPER SULPHATE UPON THE TREMOR PRODUCED BY NN-DIETHYLCYSTEAMINE

Treatment	Tremor
Water; NN-diethylcysteamine 15 min later	1, 1, 2, 0, 0, 2, 1, 1, 0, 1, 0, 1, 1, 2, 1, 1, 1, 1, 1, 1
Copper sulphate ; NN-diethylsysteamine 15 min later	2, 2, 1, 1, 1, 2, 2, 2, 2, 0, 3, 2, 3, 1, 2, 1, 3, 2, 3, 3.

Tremor was assessed 5 min after the injection of NN-diethylcysteamine using an arbitary ranking scale. A double blind procedure was used. General conditions were as Table 3.

of tremor was observed when copper was given after NN-diethylcysteamine or either before or after cysteamine (Table 3). Experiments were also done in which the copper was given together with cysteamine (111 mg/kg body weight; 10 animals) or NN-diethylcysteamine (42·5 mg/kg body weight; 8 animals). No significant effects of copper upon the tremor was noted.

DISCUSSION

The LD_{50} values found in these experiments for cysteamine and NN-diethylcysteamine injected intraperitoneally into mice are comparable to the values previously found by Koch and Hagen[1] of 247 mg/kg body weight and 120 mg/kg body weight respectively using subcutaneous injection. The toxicity of NN-diethylcysteamine was shown to be markedly potentiated by copper sulphate while the toxicity of cysteamine was only affected slightly. The turbidity observed on mixing copper sulphate with cysteamine but not with NN-diethylcysteamine suggests that the cysteamine copper complex may have been less available for transport to sites of action than the NN-diethylcysteamine-copper complex. The findings with NN-diethylcysteamine extend the original observation of Stern, Ratkovic and Fuks[2] of the enhancement of its toxicity by copper. Similarly, the toxicities of lead and bismuth salts of cysteamine injected intraperitoneally into mice were shown by Bonati[8] to be greater than the sum of the toxicities of cysteamine and other salts of the metals. Copper sulphate has a borderline potentiating effect on cysteamine toxicity.

Though enhancement of NN-diethylcysteamine tremor by copper was shown it was not possible to demonstrate an enhancement of cysteamine tremor. However, strictly comparable experiments were not possible. Due to the greater amounts of cysteamine needed to produce tremor, the copper to cysteamine molar ratio had to be kept lower than the copper to NN-diethylcysteamine molar ratio to avoid concentrations of copper at which it would have been lethally toxic alone. It must be emphasised also that the method used for assessing tremor is crude as compared with the more precise determination of LD_{50} and hence minor tremor changes could be easily missed.

The acute toxic effects of cysteamine and NN-diethylcysteamine, namely convulsions at high dosages indicate a central action of these substances. The tremor caused by NN-diethylcysteamine was also considered by Stern, Ratkovic and Fuks[2] to be of central origin, being potentiated by various centrally acting drugs. The enhanced tremor observed in mice when copper was injected intraperitoneally 15 min before NN-diethylcysteamine indicates potentiation by copper of an effect of NN-diethylcysteamine as tremor was not observed when even much larger amounts of copper alone were used. Copper has been shown to chelate very strongly with cysteamine[9] and cysteamine-copper complexes with charge-transfer properties have been reported[10] which may well indicate high biological activity.[11] While many examples are known of the enhancement of activity of metals by chelating agents,[12] the enhancement by metals of the activity of chelating ligands has received much less attention.

Little is specifically known about the biochemical properties of NN-diethylcysteamine but properties of cysteamine are known which might profitably be investigated further in relation to tremor. Thus, it is of interest that cysteamine causes catecholamine release from the adrenals,[13] and that the catecholamine dopamine potentiates NN-diethylcysteamine tremor.[2] There is some evidence that disturbed catecholamine metabolism may be causally related to the tremor states occurring in various extrapyramidal diseases (reviewed by Walaas and Walaas[14]).

If a cysteamine-like substance were demonstrated in brain then the copper potentiated tremor might be a close model of the intention tremor of Wilson's Disease. While cysteine is not decarboxylated to cysteamine by amino acid decarboxylases

other pathways of cystemine formation *in vivo* have been suggested[15] though direct, evidence is at present lacking.

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