UDC 615.733[547.466.2'156]-092

156. Hikaru Ozawa, Chuichi Ishizeki, and Munetoshi Niimura : Copper DL-Methionine, a New Anthelmintic for Swine Lung Worm. The Action of Copper DL-Methionine on Lung Worms.

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The lung worm, *Metastrongylus apri*, is a parasite that attacks the lung of swine. The rate of infection in Japan is from 50% to 80%, the infection causes growth impediment in piglets, and sometimes causes their death, so that the countermeasure for the worm is a great problem in dairy industry. At the time of starting this work, in 1956, no therapeutic was known and the only effective measure was disinfection of pig pens to remove the earthworm which is known as the intermediate host of this worm.

As a result of fundamental experiments, copper DL-methionine*3 was found to be the best anthelmintic, both from the point of efficacy and toxicity. The effectiveness of this chemical was later proved from the field work of therapeutic experiments by Tamazaki, *et al*,[2,3] and others.[3] As a part of fundamental experiments, the action of copper DL-methionine on this lung worm will be described in this paper.

Experimental

Anthelmintic Test *in vitro*——There is no report on the method of testing anthelmintic effect against the lung worm by *in vitro* test and examination was first made on the culture of the worm in a vessel. Fifteen lung worms were placed in a petri dish, 10 cc. of culture solution was added, and this was incubated at 27~28° to observe their survival period. The culture liquid was changed every 24 hr. This result is given in Table I. The culture solution of 0.5% peptone-Tyrode solution was found to be the best and addition of dehydroacetic acid as an antiseptic was found to be injurious to worms.

Table I. Average Survival Time of Lung Worms in Various Culture Solutions

Culture solution	Average survival time (hr.)
0.9% NaCl solution	30.3
Ringer solution	75.3
Locke solution	77.0
Tyrode solution	68.7
0.5% Peptone-Tyrode soln.	77.7
0.5% Peptone-Tyrode soln. with DHA* (100 γ/cc.)	68.0
0.5% Rabbit serum + 0.9% NaCl solution	60.3

* DHA=Dehydroacetic acid

Anthelmintic effect of various chemicals was tested with this culture solution and Hg^{2+} and Cu^{2+} were found to be the most effective, as shown in Table II.

Of these chemicals tested, Hg^{2+} is too toxic, so that examination was further made on Cu^{2+} compounds. Copper citrate, nicotinate, ethylenediaminetetraacetate, glycolate, L-aspartate, and copper DL-alanine and L-valine were tested, either as aqueous solution or, in sparingly soluble compounds, as suspension in 1% carboxymethyl cellulose solution. Survival time was 18~20 hr. in all these compounds, far shorter than that of 72 hr. in the control.

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*3 Japanese Patent No. 35-7297 (1960).

1) H. Ozawa, C. Ishizeki, *et al.* : J. Vet. Med., **208**, 619 (1957).
2) K. Tamazaki, H. Ozawa, *et al.* : *Ibid.*, 213, 920 (1957).
3) S. Tsunohashi : Nippon Juishikai Zasshi, **13**, 23 (1960).

Table II. Anthelmintic Activity of Various Compounds on Lung Worms in Culture Solutions

Compound	Concn.	Average survival time (hr.)
Control	—	72.0
$CoSO_4 \cdot 7H_2O$	1 : 5000	93.0
$MnSO_4 \cdot 7H_2O$	1 : 5000	90.0
$ZnCl_2$	1 : 5000	80.0
$SeO_2 \cdot xH_2O$	1 : 5000	58.0
$HgCl_2$	1 : 100000	1.4
$CuSO_4 \cdot 5H_2O$	1 : 100000	16.0～18.0
Copper DL-methionine	1 : 100000	16.0～18.0
Copper L-glutamate	1 : 100000	16.0～18.0
CCl_4	1 : 5000	41.0
$CHCl_3$	1 : 5000	30.0
$CNCH_2CONHNH_2$	1 : 100000	50.0
Piperazine hydrate	1 : 5000	39.0

Relationship between the concentration and anthelmintic effect was examined with $CuSO_4$, copper DL-methionine, and copper sodium L-glutamate. It is interesting, as shown in Table III, that there was no marked shortening of survival time according to the concentration.

Table III. Anthelmintic Action of High Concentrations of Copper Compounds on Lung Worms

Concn. of Cu^{2+}	Survival time (hr.)		
	$CuSO_4 \cdot 5H_2O$	Copper DL-methionine	Copper L-glutamate
1 : 1000	16.0～18.0	—	—
1 : 5000	16.0～18.0	10.0	10.0
1 : 10000	16.0～18.0	16.0	15.0
1 : 50000	16.0～18.0	16.0	15.0
1 : 100000	16.0～18.0	20.0	25.0
1 : 500000	20.0	43.0	36.0
Control	72.0	72.0	72.0

Uptake of Copper by Worms——In order to find the route of the penetration of Cu^{2+} into the worm, *in vitro* test was carried out on the worms, with a difinite concentration of copper DL-methionine, and a worm was taken out at definite intervals. The worm was made into frozen sections, stained with rubeanic acid solution (5 cc. of 1% rubeanic acid and 1000 cc. of 10% AcONa), which selectively stains Cu^{2+}, and distribution of Cu was examined. As shown in Figs. 1 and 2, Cu was localized in the digestive tract and moved gradually into lower portions with lapse of time. Since Cu was not found in any part of body surfaces, it was considered that Cu^{2+} is taken in from the mouth and acts toxic internally.

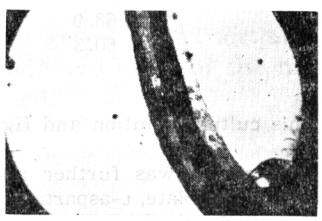

Fig. 1. Distribution of Cu in lung worm

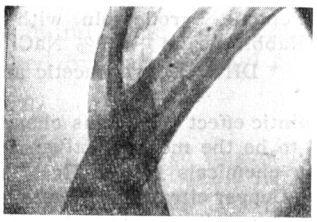

Fig. 2. Distribution of Cu in lung worm

Effect of Cu^{2+} on Worm Respiration——The lung worm lives in the lung with high partial O_2 pressure and respiration is considered to be very active. Therefore, effect of copper DL-methionine on the respiration of worms was examined.

O_2 uptake and CO_2 output were measured by the Warburg manometric technique, using intact worms and worm homogenate. Into the manometric flask, 3.2 cc. of 0.5% peptone-Ringer solution was placed, 10 intact lung worms or their homogenate was added, and O_2 uptake or CO_2 output was measured every 10 min. Both were more active in the intact worms and respiration quotient (RQ)

after 120 min. was 35.6 μL./74.4 μL.=0.51 in intact worm and 9.8 μL./21.0 μL.=0.41 in the homogenate.

Table IV gives the Q_{O_2} (O_2 uptake of 1 mg. of dry worm/hr.) and Q_{CO_2} (CO_2 output of 1 mg. of dry worm/hr.), which are far greater than those of other parasitic worms. This fact is thought to indicate the great ability of lung worms to take up O_2, especially the development of respiration enzyme, by living in O_2-rich sites.

Measurement of Q_{O_2} and Q_{CO_2} during application of copper DL-methionine (1 : 5000) to intact lung worms and their homogenate showed that respiration is inhibited to a fairly great extent.

Table IV. Q_{O_2} and Q_{CO_2} of Lung Worms and the Effect of Copper DL-Methionine on Respiration

Parasitic worm	Q_{O_2}		Q_{CO_2}	
	Control	Cu (1 : 5000)	Control	Cu (1 : 5000)
Metastrongylus apri				
Intact	5.90	4.60	4.43	3.10
Homogenate	1.51	0.90	1.03	0.78
Fasciola hepatica (Liver fluke)				
Intact	1.32		2.20	
Ascaris lumbricoides	1.94[a]			
Intact	0.32[b]			
	0.59[c]			

a) van Grembergen : Enzymologia, **13**, 241 (1949).
b) W. Adams : Z. vergleich. Physiol., **16**, 229 (1932).
c) von Brand : *Ibid.*, **21**, 220 (1934).

Inhibitory Action of Cu^{2+} on Succinic Dehydrogenase——The highly developed respiratory functions of the swine lung worms suggest high activity of respiration enzyme and the effect of Cu^{2+} on respiratory enzyme system and succinic dehydrogenase was examined.

(1) By the use of Methylene Blue : Thunberg tube was used for this test, 1 cc. of lung worm homogenate solution (250 mg. of the homogenate) and 2 cc. of dist. H_2O were added to the main chamber, and 1 cc. of 0.1*M* sodium succinate and various concentrations of Methylene Blue solution were added to the side arm. The two solutions were mixed, shaken, and reacted in a thermostat of 37°, to measure the time of discoloration. The time required until discaloration became longer as the concentration of Methylene Blue became higher and discoloration in 10～30 min. was found to be effected with 0.001*M* Methylene Blue (cf. Table V).

Table V. Reducing Time of Methylene Blue by Lung Worm Homogenate

Metylene Blue concn. (*M*)	0.005	0.0025	0.001	10^{-4}	7×10^{-5}	3×10^{-5}	10^{-5}	3×10^{-6}
Reducing time	32′30″	22′20″	13′30″	3′00″	1′25″	1′00″	0′30″	0

Under these conditions, prolongation of reducing time of Methylene Blue was measured with addition of copper DL-methionine (1 : 50000 and 1 : 10000) and it was revealed that Cu^{2+} markedly inhibited succinic dehydrogenase activity, as shown in Table VI.

Table VI. Inhibitory Effect of Cu^{2+} on Succinic Dehydrogenase of Lung Worm, measured by Methylene Blue Discoloration

Concn. of copper DL-Methionine	Reducing time
Nil (Control)	11′30″
1 : 50000	27′20″
1 : 10000	42′30″
1 : 5000	180′00″

(2) By the use of 2,3,5-triphenyl-2*H*-tetrazolium chloride : The same test was carried out with the Thunberg tube, using 2,3,5-triphenyl-2*H*-tetrazolium chloride (T.T.C.) to measure triphenylformazan formed and examine the inhibitory action of Cu^{2+} on succinic dehydrogenase of the lung worm. As shown in Table VII, succinic dehydrogenase of lung worm homogenate reduces 0.072 mg. of T.T.C. by contact for 30 min., while addition of copper DL-methionine (1 : 10000) decreased this to 0.039 mg., an inhibition of 46%.

Table VII. Inhibitory Effect of Cu^{2+} on Succinic Dehydrogenase of Lung Worm

Compound	Concn. (*M*)	Wt. of reduced T.T.C. (mg.)
Control	—	0.072
KCN	0.1	0.071
Sodium malonate	0.01	0.070
Sodium malonate	0.02	0.040
Copper DL-methionine	1 : 10000	0.039

Discussion

Copper (II) ion was found to have a powerful lethal action on swine lung worms and attempt was made to elucidate its action mechanism.

Since the final parasitic site of this worm is the lung trachea rich in oxygen, oxygen consumption of this worm was found to be much greater than other worms. Copper (II) ion showed inhibitory action against this, especially against succinic dehydrogenase which is closely related to respiration. This inhibition of respiration may be the main reason for the anthelmintic action of copper but since the compound also inhibits succinic dehydrogenase action of other parasites, this fact does not explain the selective lethal action of copper (II) ion on the swine lung worm, although it could be one of several factors.

Summary

Examination of various compounds effective aginst lung worm parasitic to swine and a great problem in dairy industry showed that copper DL-methionine is an effective agent, both from fundamental experiments and clinical test, and has little toxicity. In the present series of experiment, *in vitro* culture of the lung worms was attempted and anthelmintic effect of various compounds was tested by this *in vitro* culture, Copper (II) ion was found to be specifically effective. Copper (II) ion is absorbed through the digestive tract of the worm to become toxic and is not absorbed through the body surface. Effect of copper (II) ion is lowering of respiration and one of its reasons was thought to be the marked inhibition of succinic dehydrogenase action of the worms.

The lung worm lives in the swine lung rich with oxygen and its oxygen consumption is great so that its inhibition proves lethal to the worm.

(Received May 13, 1960)