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The action of allyl-, phenyl-isothiocyanate and bithionol on glycolytic and oxidative metabolism in the larvae of a nematode, *Anisakis* sp.

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Introduction

From the medical point of view, *Anisakis* infection has been recognized to be highly important. Up to the present, more than 440 cases diagnosed as gastric polyposis peptic ulcer, gastritis, etc., which are considered to be caused by the *Anisakis* larvae, have been reported in Japan (Kobayashi, 1967 ; Otsuru, 1968). Very little, however, is so far known concerning information on the prevention and medical treatment of *Anisakis* infection.

The authors have already reported that the larvicidal action of Japanese horseradish powder (Wasabi) and isothiocyanates against *Anisakis* larvae was effective *in vitro* (Kawashima & Hamajima, 1966). On the other hand, it has been recognized that bithionol is the most effective drug to treat for paragonimiasis (Yokogawa *et al.*, 1961 a b). Thereafter, the present authors proved that this drug was effective to kill *Anisakis* larvae *in vitro* (unpublished). Then, the present study was carried out, by the use of these drugs —— isothiocyanates and bithionol ——, to make clear whether the glycolytic and oxidative metabolism of *Anisakis* larvae which was reported in the previous paper (Hamajima *et al.*, 1969) was interfered or not. In the present paper, the authors wish to report the results of the experiments with some comment.

Materials and Methods

Anisakis larvae were obtained from the internal organs of freshly killed horse mackerels (*Trachurus japonicus*) that were captured in East China Sea. This nematode was identical with type I of *Anisakis* larvae (Oshima, 1966 ; Kobayashi, 1967). Preparation of the materials was performed in the same procedure as described in the previous report (Hamajima *et al.*, 1969). Homogenates were prepared with the aid of a motor-driven glass homogenizer in an ice bath for 5 minutes, by using various kinds of medium depend on the experiment.

Lactate produced by the homogenate of the *Anisakis* larvae was estimated by the method of Barker & Summerson (1941). Oxygen uptakes were determined by the conventional Warburg technique (Umbreit *et al.*, 1951). Succinate dehydrogenase activity was examined by visual measurement of 90% methylene blue reduction by employing the Tunberg technique (Umbreit *et al.*, 1951). Cytochrome oxidase activity was essentially determined by the spectrophotometric method of Cooperstein & Lazarow (1951). In this experiment, the homogenate in 0.03 *M* phosphate buffer (pH 7.4) was centrifuged at 3,000 r.p.m. for 5 minutes. The supernatant fluid was used. The nitrogen content of the preparations was determined by a micro-

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Kjeldahl procedure. Allyl- and phenyl-isothiocyanate were purchased from the Tokyokasei Chemical Co., Tokyo. Bithionol was a gift from the Tanabe pharmaceutical Co., Osaka. The soluble bithionol was prepared from bithionol by neutralization with KOH. All reagent employed were of the highest available purity.

Results and Discussion

1. Effect of allyl-, phenyl-isothiocyanate and bithionol on the glycolytic metabolism.

As shown in Table 1, 0.01 M allyl-isothiocyanate inhibited the lactate formation below the level of the complete system in all cases and $8\times10^{-3}\,M$ phenyl-isothiocyanate inhibited the formation when glucose was used as the substrate. The result was similar to that of the experiment on glycolysis in various parasites inhibited by some drugs (Bueding, 1949, 1950; Mansour & Bueding, 1954; Bueding & Mansour, 1957, Bueding *et al.*, 1947; Murakoshi *et al.*, 1962; Bryant *et al.*, 1963; Yokogawa, 1964; Mattila & Takki, 1966; Hamajima, 1966, 1968 ab). On the contrary, the phenyl-isothiocyanate stimulated the formation above the level of the complete system when glycogen or fructose-1, 6-diphosphate (FDP) was used as the substrate. The identical result was obtained by Mansour (1959) and Mansour & Menard (1960) in the case of effect of serotonin on the glycolysis of *Fasciola hepatica*. In the cases, it appears that glycolysis proceeded at an exceeding rate.

Previously, the effect of some Japanese seasonings against *Anisakis* larvae was examined, and it was elucidated that the larvicidal action of Japanese horseradish (wasabi) powder was the most effective (Kawashima & Hamajima, 1966). Yasuda (1957) found that the larvicidal action of mustard oil was the most effective against the hook worm larvae. Kojima *et al.* (1966) detected allyl- and phenyl-isothiocyanate as the acrid components of commercial wasabi powder by gas chromatographic analysis. Thus, the experiments described above suggested that the larvicidal action of wasabi powder might depend on the isothiocyanate which is one of the main content of the wasabi powder. Then, the experiments were carried out, by the use of pure isothiocyanates, to find out whether the larvicidal action observed with wasabi powder can be attributed to isothiocyanates. Consequently, it was elucidated that the larvicidal action of allyl- and phenyl-isothiocyanate against the *Anisakis* larvae was strong (Kawashima & Hamajima, 1966), and that both isothiocyanates interfered with the reaction of glycolytic and oxidative metabolisms in *Anisakis* larvae (Hamajima & Kawashima, 1966).

As shown in Table 1, $10^{-4}\,M$ bithionol markedly stimulated the lactate formation above the level of the complete system when glycogen or glucose was used as the substrate. On the contrary, bithionol inhibited the formation below the level of the complete system when FDP was used as the substrate.

Yokogawa *et al.* (1961 a) found that bithionol had remarkable potency to kill *Paragonimus westermani in vitro* as well as in animal experiments, and also Yokogawa *et al.* (1961 b) reported that bithionol was the most effective drug for the chemotherapy of paragonimiasis. In addition, Sawatari & Hamajima (1967) found that bithionol was effective for destruction of adults and metacercariae of *P. westermani in vitro*. On the other hand, it was evident that in *Paragonimus* bithionol inhibited the glycolysis and oxidative metabolism (Murakoshi *et al.*, 1962; Yokogawa, 1964; Hamajima, 1966). From the standpoint of medical treatment, the effect of bithionol against *Anisakis* larvae was examined *in vitro* by the present authors, and it was proved that $10^{-4}\,M$ bithionol killed all the larvae within 24 hours after treatment (unpublished data). More recently, as the glycolytic and oxidative metabolism had been demonstrated to occur in homogenate of *Anisakis* larvae (Hamajima *et al.*, 1969), it would be of interest to point out that the metabolism of *Anisakis* larvae is sensitive to the inhibitory effect of bithionol as found in *Paragonimus* by Murąkoshi *et al.* (1962),

Table 1 Effect of allyl-, phenyl-isothiocyanate and bithionol on the lactate formation by the homogenate of *Anisakis* larvae

Substrate	Reaction mixture	Lactic acid produced	
		μ moles	QL(N)
Glycogen	Complete	17.56	19.19
	Plus allyl-isothiocyanate (15 μ moles)	6.17	6.74
	Plus phenyl-isothiocyanate (12 μ moles)	23.09	25.24
	Minus glycogen	15.79	17.26
Glucose	Complete	17.56	19.19
	Plus allyl-isothiocyanate (15 μ moles)	2.12	2.32
	Plus phenyl-isothiocyanate (12 μ moles)	4.25	4.65
	Minus glucose	5.42	5.92
F D P	Complete	31.97	34.94
	Plus allyl-isothiocyanate (15 μ moles)	27.83	30.42
	Plus phenyl-isothiocyanate (12 μ moles)	40.06	43.79
	Minus FDP	19.34	21.14
Glycogen	Complete	8.91	9.74
	Plus bithionol (0.15 μ mole)	47.82	52.27
	Minus glycogen	1.07	1.17
Glucose	Complete	4.75	5.19
	Plus bithionol (0.15 μ mole)	36.17	39.53
	Minus glucose	1.07	1.17
F D P	Complete	40.75	44.54
	Plus bithionol (0.15 μ mole)	28.27	30.90
	Minus FDP	1.07	1.17

The complete system contained 60 μ moles of KH_2PO_4 (pH 7.2 with KOH), 15 μ moles of $MgCl_2$, 45 μ moles of KCl, 1.5 μ mole of ATP, 0.15 μ mole of NAD, 60 μ moles of nicotinamide, 0.3 μ mole of FDP*, substrate (0.15 ml of 3 % glycogen, 90 μ moles of glucose or 75 μ moles of FDP), 1.5 μl of polyethylene glycol* and 0.45 ml of 25 % homogenate in 0.03 *M* phosphate buffer (pH 7.2) and water in final volume of 1.5 ml. Reactions were run for 60** or 120* minutes at 37°C *in vacuo*. The amounts of lactate production are shown in μ moles lactic acid produced per gram in wet weight per hour. QL(N) is μl gas volume released under standard condition from lactic acid produced per milligram nitrogen per hour. The values presented are the averages of triplicate determinations.

* This mark indicates the cases in the experiment of isothiocyanates.

** This mark indicates the cases in the experiment of bithionol.

Yokogawa (1964) and Hamajima (1966). The action of bithionol on the metabolism in *Anisakis* larvae was examined in this experiment. Consequently, the results were in agreement with the observations of Murakoshi *et al.* (1962), Yokogawa (1964) and Hamajima (1966) who reported that bithionol interfered with the glycolysis and the tricarboxylic acid cycle of *P. westermani*.

2. Effect of allyl-, phenyl-isothiocyanate and bithionol on the oxidative metabolism.

The results were summarized in Tables 2, 3 and Figure 1. As shown in Table 2, 0.01 *M* allyl-, 8×10^{-3} *M* phenyl-isothiocyanate and 10^{-4} *M* bithionol inhibited the oxygen consumption below the levels of the complete system and endogenous. The result obtained in this study was similar to that the oxygen

consumption in various parasites was inhibited some drugs (Bueding, 1949; Bueding *et al.*, 1947; Massey & Rogers, 1951; Murakoshi *et al.*, 1962; Ozawa *et al.*, 1962; Yokogawa, 1964; Hamajima, 1966, 1968 a b and Hamajima & Sawatari, 1966).

As shown in Table 3, succinate stimulated the reduction of methylene blue by the homogenate. However, $6\times10^{-3}\,M$ malonate inhibited the reduction of methylene blue. The addition $0.01\,M$ allyl- or $8\times10^{-3}\,M$ phenyl-isothiocyanate inhibited the reduction of methylene blue below the level of the complete system. It appeared that succinate dehydrogenase was inhibited by allyl- and phenyl-isothiocyanate. Furthermore, the succinate dehydrogenase in *P. westermani* was inhibited by bithionol (Murakoshi *et al.*, 1962;

Table 2 Effect of allyl-, phenyl-isothiocyanate and bithionol on the oxygen consumptions in the homogenate of *Anisakis* larvae

Substrate	Reaction mixture	O_2 uptake	
		μ l	QO_2(N)
Succinate	Complete	155.60	7.59
	Plus allyl-isothiocyanate (30 μ moles)	99.25	4.84
	Plus phenyl-isothiocyanate (24 μ moles)	87.70	4.28
	Minus succinate	110.25	5.38
Succinate	Complete	137.58	6.71
	Plus bithionol (0.3 μ mole)	73.96	3.61
	Minus succinate	95.78	4.67

The complete system contained 30 μ moles of KH_2PO_4 (pH 7.3 with KOH), 12 μ moles of $MgCl_2$ and $NnCl_2$, 3 μ moles of ATP, 0.3 μ mole of NAD and NADP, 60 μ moles of nicotinamide, 30 μ moles of succinate, 485 μ moles of sucrose, 3 μl of polyethylene glycol*, 1.0 ml of 20** or 25+ % homogenate in 0.25 M sucrose and water in final volume of 3.0 ml. The gas phase was air. Reactions were run for 60* or 120** minutes after equilibration at 37°C. The quantities of oxygen consumption are shown in μl oxygen consumed per gram in wet weight per hour. QO_2(N) is μl oxygen consumed per milligram nitrogen per hour. The values presented are the averages of triplicate determinations.

** These marks are the same as in Table 1.

Table 3 Effect of succinate, allyl- and phenyl-isothiocyanate on methylene blue reduction by the homogenate of *Anisakis* larvae

Substraate	Reaction mixture	Time for 90 % reduction (minutes)	% Decrease due to addition
Succinate	Complete	10	95
	Plus phenyl-isothiocyanate (32 μ moles)	15	92
	Plus allyl-isothiocyanate (40 μ moles)	20	89
	Plus malonate (24 μ moles)	25	86
	Minus succinate	180	—

The complete system contained 0.267 μ mole of methylene blue, 40 μ moles of succinate, 4 μl of polyethylene glycol, 1.0 ml of 10 % homogenate in M/15 phosphate buffer (pH 7.2) and M/15 phosphate buffer (pH 7.2) in final volume of 4.0 ml. Reactions were run at 37°C *in vacuo*.

Yokogawa, 1964; Hamajima, 1966) and isothiocyanates (Hamajima & Sawatari, 1966). On the contrary, Tamura (1962) reported that kainic acid did not extremely inhibit the succinate dehydrogenase of *Ascaris lumbricoides suum*.

As shown in Figure 1, the reduced cytochrome c was oxidized by the supernatant fluid, and the oxidation of reduced cytochrome c was inhibited by 0.01 *M* sodium cyanide to reaction mixture. The addition of 0.01 *M* allyl- and 8×10^{-3} *M* phenyl-isothiocyanate inhibition the oxidation of reduced cytochrome c by the supernatant fluid. This was in agreement with the observation of Hamajima & Sawatari (1966) who reported that allyl- and phenyl-isothiocyanate inhibited the oxidation of reduced cytochrome c in *P. westermani*.

On the basis of the result mentioned above, it was recognized that allyle- and phenyl-isothiocyanate inhibited the activities of both succinate dehydrogenase and cytochrome oxidase as shown in Table 3 and Figure 1. Thus, it appeared that both isothiocyanates inhibited the succinoxidase system of *Anisakis* larvae. Judging from these results, it was suggested that isothiocyanates or bithionol interfered with the oxidative metabolism of the *Anisakis* larvae.

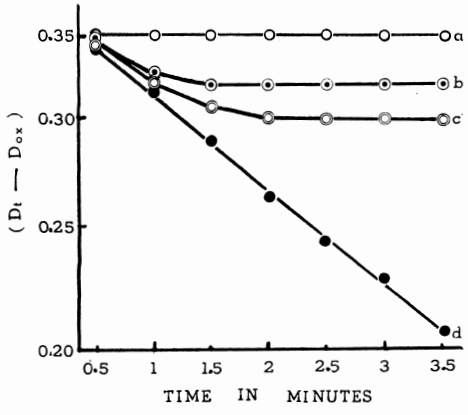

Fig. 1 Graph (on log scale) showing the effect of sodium cyanide, allyl- and phenyl-isothiocyanate on the oxidation of ferrocytochrome c by the supernatant fluid of the homogenate of *Anisakis* larvae.

The complete system contained 0.17 μ mole of cytochrome c, 4 μ l of polyethylene glycol, 0.2 ml of supernatant fluid of 10% homogenate in 0.03 *M* phosphate buffer (pH 7.4) and 0.03 *M* phosphate buffer (pH 7.4) in final volume of 4.0 ml. Reactions were run at 21°C. Dt is the extinction at time t. Dox is the extinction after complete oxidation by the addition of potassium ferricyanide (4 μ moles). Assay time is 3.5 minutes.

a Plus sodium cyanide (40 μ moles). b Plus phenyl-isothiocyanate (32 μ moles). c Plus allyl-isothiocyanate (40 μ moles). d Complete system.

Summary

In the present study, from the standpoint of the prevention and medical treatment for *Anisakis* infection, the effect of allyl-, phenyl-isothiocyante and bithionol on glycolytic and oxidative metabolism was investigated in the homogenate of *Anisakis* larvae.

1. Allyl-isothiocyanate extremely inhibited the formation of lactate below the level of the complete system in all cases, and phenyl-isothiocyanate inhibited the formation when glucose was used as the substrate, and also bithionol inhibited the formation when FDP was used as the substrate. On the contrary, phenyl-isothiocyanate markedly stimulated the formation of lactate when glycogen and FDP was used as the substrate and bithionol stimulated the formation when glycogen and glucose was used as the substrate.

2. The same drugs inhibited the oxygen consumption below the level of the complete system and endogenous.

3. Allyl- and phenyl-isothiocyanate inhibited the reduction of methylene blue and the oxidation of reduced cytochrome c.

From the reason mentioned above, allyl-, phenyl-isothiocyanate and bithionol interfered with glycolytic and oxidative metabolism in the *Anisakis* larvae. Because of these facts, it seemed to show that allyl-, pheny-isothiocyanate and bithionol may potentially be useful for control of *Anisakis* infection.

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アニサキス幼虫における解糖および呼吸代謝に対する allyl-, phenyl-isothiocyanate および bithionol の作用

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アニサキス成虫はマッコウクジラおよびスジイルカ等の海産哺乳動物に寄生しているが，その幼虫は人体消化管内に肉芽腫を形成することが知られている．今日までに，日本においてその幼虫によるとみられる症例報告は440例以上に達している．しかし，われわれの知るかぎりでは薬による本症の予防および治療に関する研究はきわめて少ない．そこで，アニサキス症の予防および治療の基礎的研究として，その解糖および呼吸代謝に対するallyl- および phenyl-isothiocyantae と bithionol の作用を検討した．その結果，allyl-isothiocyanate はすべての場合，乳酸形成量を抑制し，また，phenyl-sothiocyanate はグルコースが，bithionol は FDP が基質として用いられたときにその形成量を抑制した．これに対して，phenyl-isothiocyanate はグリコーゲンと FDP が，bithionol はグリコーゲンとグルコースが基質として用いられたときにその形成量を刺激した．一方allyl-, phenyl-isothiocyanate および bithionol はホモジネートの酸素消費量を抑制した．また，allyl- および phenyl-isothiocyanate はホモジネートによるメチレンブルーの還元を抑制し，ホモジネートの上清による還元チトクローム C の酸化を抑制した，以上のことから，allyl-phenyl-isothiocyanate および bithionol はそれぞれこの幼虫の解糖および呼吸代謝を阻害し，アニサキス症の予防と治療に効果のある可能性をした．