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STUDIES ON ECHINOCOCCOSIS XXV

ANTHELMINTIC ACTION OF DRUGS ON LARVAL *ECHINOCOCCUS MULTILOCULARIS* IN VITRO*

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The protoscolices and free cysts of *Echinococcus multilocularis* were incubated for ten days in three kinds of media to which different kinds of the drugs were added at the rates of 10, 40 and 100 γ per ml. The main medium, which consists essentially of medium 199 with calf serum in proportion of 20 per cent, was used for the drugs soluble in water. Most drugs insoluble or slightly soluble in water were added to the main medium as a solution in 2 mg per ml of propylene glycol. To observe the effect of propylene glycol, the scolicidal effect of bunamidine hydrochloride suspended in the medium containing 0.3 % methylcellulose were compared with that in the medium with propylene glycol. The survival rates of protoscolices and free cysts with each drug were compared to each other together with those tested in the preceding report (SAKAMOTO et al., 1965).

The derivatives of halogenized salicylanilide manifested the highest scolicidal effect, and the derivatives of halogenized bisphenol ranked next. Bunamidine hydrochloride and cyanine dyes gave a comparatively high scolicidal effect. The intensity of scolicidal action in halogenized derivatives of salicylanilide and bisphenol increased with the increase of the number of halogen atoms, such as chlorine and bromine, in their phenol rings. The presence of propylene glycol in the medium shows a tendency to increase the intensity of scolicidal action of drugs insoluble in water.

INTRODUCTION

Chemotherapy has been considered to be great value in the treatment of those suffering from echinococcosis, particularly in patients who suffer from multilocular echinococcosis which is inoperable. Correspondingly, for many years, it has been a dream of investigators to discover a drug effective against echinococcosis. LEHMANN (1928) reviewed historically chemotherapies for echinococcosis. According to the review, the inunction of mercurial ointment, iodine ointment and tincture of iodine, the injection of arsenobenzol, and the oral

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administration of an iodine preparation, a quinine preparation, tincture of kamala and other cestocidal chemicals were carried out empirically by practitioners. To eliminate the risk of secondary infection, on the other hand, tincture of iodine, alcohol, corrosive sublimate, iodoform glycerin, formalin, formalin glycerin and phenol were infused into echinococcal cysts prior to their surgical removal. However, no available evidence on the efficacy of the above-mentioned drugs against echinococcal larval tissue has heen confirmed by investigators. The drugs infused into the cyst rather frequently caused risk to the host because of the accidental spillage into the serous cavity.

Recently, UNO et al. (1956) and MIKAMI et al. (1956) used trivalent antimonial and pentavalent antimonial in the treatment of multilocular echinococcosis in humans, respectively. HANSTEIN (1957), YAMADA et al. (1962) and YAMADA (1962, 1963) reported the chemotherapeutic efficacy of palmitic acid thymol ester on multilocular echinococcosis in humans. However, YAMASHITA (1961) and YAMASHITA et al. (1962) reported that the above three drugs were ineffective against experimental multilocular echinococcosis in mice. In their examinations, particularly, the injection of oily solution of palmitic acid thymol ester, which was reported by HANSTEIN (1957), YAMADA et al. (1962) and YAMADA (1962, 1963) to give the therapeutic efficacy against human multilocular echinococcosis, resulted in a tendency to accelerate the development of echinococcal tissue in mice. They observed that most of the oily solution injected was not absorbed from the region where it was infused. In recent years, LUBINSKY & GALAUGHER (1967), LUBINSKY (1969, 1970) and LUBINSKY et al. (1971) have tested an inhibitive effect of many drugs including cytostatic agents on the growth of vegetatively propagated *E. multilocularis* in mice. They observed that five cytostatic agents and an antischistosomal agent inhibited the growth of the cysts, but only when used in toxic doses. No drug, therefore, has been established as an effective chemotherapeutic agent against echinococcosis.

As regards the in vitro screening test, CLUNIES ROSS (1927) reported the action of four anthelmintics on *E. granulosus* protoscolices in vitro at first. After that, the in vitro assessment for various drugs on the protoscolices were followed by SCHWABE et al. (1963), MEYMERIAN et al. (1963), LAMY (1963, 1965), HESLOP (1967), PANAITESCO (1968) and FRAYHA et al. (1971). On the other hand, SAKAMOTO et al. (1965) tested in vitro the cytopathic effect of seven drugs on protoscolices and daughter cysts of *E. multilocularis*. And LUKASHENKO et al. (1970, 1971) assessed the in vitro inhibitory influence of eighteen compounds upon the respiration of free and encysted protoscolices of *E. multilocularis*.

The preceding report (SAKAMOTO et al., 1965) dealt with the water soluble compounds. In the present paper, the result of tests with drugs including

compounds insoluble and slightly soluble in water are presented. The relationship between the chemical structure and the efficacy of drugs is discussed together with the results of the preceding report.

Materials and methods

Larval echinococcal tissues used in the present experiment were prepared and cultured in the same manner as those in the preceding report. Namely, protoscolices and free daughter cysts were collected aseptically from the liver of cotton rats experimentally infected with *Echinococcus multilocularis*, echinococcal tissue containing abundant protoscolices were treated with 0.2% trypsin solution, and the protoscolices and free cysts released were washed several times with Hanks' solution. Medium 199 (Morgan et al., 1950) was used with 20% calf serum previously inactivated at 56°C for 30 minutes and Millipore filtered. To the medium 100 units of penicillin G and 100 γ of streptomycin sulfate per ml were added. Cubic culture bottles (250 ml) containing 15 ml of medium with about 10,000 protoscolices or about 1,000 free cysts were kept at 37°C. The pH of the culture medium indicated by phenol red was adjusted at 7.4 with 2.8% $NaHCO_3$ every day. The medium was changed every other day.

The present screening test to the following drugs were carried out: gentian violet and pyrvinium pamoate as compounds of cyanine dyes, sodium antimony gluconate of antimonial, acetarsone (*N*-acetyl-4-hydroxy-*m*-arsanilic acid) of arsenical, piperazine adipate, methalofyne (3-methyl-1-pentyn-3-yl acid phthalate: Whipcide, Pitman-Moore Co.), arecoline hydrobromide, disophenol (2, 6-diiodo-4-nitrophenol: Ancylol, Lederle Co., Ltd.), chlorothymol, thymol, thymol iodide, thymol-β-D-glucoside and thymol monosuccinate as derivatives of phenol, dichlorophen, 2, 2′-methylenebis (4-chloro-6-nitrophenol) and hexachlorophen as derivatives of diphenylmethane, 2, 2′-thiobis (4-chlorophenol), bithionol (2, 2′-thiobis-(4, 6-dichlorophenol): Bitin, Tanabe Seiyaku Co., Ltd.), bithionol-sulfoxide (2, 2′-sulfinylbis (4, 6-dichlorophenol): Bitin-S, Tanabe Seiyaku Co., Ltd.), 2, 2′-thiobis-(3, 4, 6-trichlorophenol), disodium-thiobis (3, 4, 6-trichlorophenolate), 2, 2′-thiobis-(4-chloro-6-nitrophenol) (DS-6, Showa Yakuhin Kako Co., Ltd.), disodium-thiobis-(4-chloro-6-nitrophenolate) and 4, 4′-thiobis (2, 6-dichlorophenol) as derivatives of diphenol sulfate, thiabendazole (2-(4-thiazolyl) benzimidazole: Thibenzole, Merk & Co., Inc.), tetramisole (*dl*-2, 3, 5, 6-tetrahydro-6-phenylimidazo [2, 1-b] thiazole), bunamidine hydrochloride (*N*: *N*-di-*n*-butyl-4-hexyloxy-1-naphthamidine hydrochloride: Scolaban, Wellcome Foundation Ltd.), 3, 5-dibromosalicylanilide, 3, 5, 4′-tribromosalicylanilide, 3, 5, 4′-trichlorosalicylanilide, 3, 5, 3′, 5′-tetrachlorosalicylanilide and niclosamide (2′, 5-dichloro-4′-nitrosalicylanilide: Yomesan, Bayer Pharmaceutical Co., Ltd.) of salicylanilide derivative, niridazole (1-(5-nitro-2-

TABLE 1 *Effect of drugs upon the rates of survival protoscolices in vitro*

(unit: %)

DRUG	DOSE (γ/ml)														
	10					40					100				
	DAY OF INCUBATION					DAY OF INCUBATION					DAY OF INCUBATION				
	2	4	6	8	10	2	4	6	8	10	2	4	6	8	10
Control	99.5	98.7	97.8	96.1	94.5	99.5	98.7	97.8	96.1	94.5	99.5	98.7	97.8	96.1	94.5
Gentian violet	56.7	49.7	0.2	0	0	30.1	0.7	0	0	0	0	0	0	0	0
Sodium antimony gluconate	86.6	50.3	32.7	23.2	20.0	82.3	41.5	21.9	19.2	17.6	61.8	31.1	2.5	2.4	0
Acetarsone	96.6	76.4	60.7	57.2	54.2	92.0	66.0	53.0	47.9	42.1	85.5	50.2	42.0	40.3	37.0
Piperazine adipate	96.9	28.2	14.7	11.7	10.6	96.4	19.5	11.9	10.9	10.0	84.0	13.3	9.6	8.7	6.2
Niridazol	49.2	31.9	30.3	8.7	0	40.1	17.0	0	0	0	22.6	0	0	0	0
Methalofyne	86.5	83.3	72.8	61.8	44.0	81.7	79.0	70.4	59.0	38.5	78.9	78.0	68.4	51.2	32.9
Arecoline hydrobromide	87.6	63.5	53.0	49.8	47.4	86.2	55.1	49.1	48.8	46.9	84.4	51.5	44.8	37.7	34.8
Disophenol	95.0	73.0	66.0	60.2	37.2	84.6	23.5	14.6	4.0	0	7.5	0	0	0	0
Disodium-thiobis (3, 4, 6-trichlorophenolate)	13.5	10.3	0	0	0	0.1	0	0	0	0	0	0	0	0	0
Disodium-thiobis (4-chloro-6-nitrophenolate)	19.7	12.9	0	0	0	12.0	0	0	0	0	0	0	0	0	0
Control (containing propylene glycol)	98.1	95.4	94.1	91.7	88.6	98.7	94.9	86.6	84.9	79.3	97.9	84.1	75.5	74.1	57.9
Bunamidine hydrochloride	15.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexachloroethane	70.6	28.8	27.6	23.5	17.8	37.8	4.6	2.9	2.2	1.5	9.4	1.6	0	0	0
Pyrvinium pamoate	76.9	10.4	0.3	0.2	0.1	68.1	0.2	0	0	0	61.2	0	0	0	0
Thymol	89.8	76.8	40.3	25.9	20.9	68.0	37.9	11.0	2.7	0	20.6	2.3	0	0	0
Chlorothymol	98.8	90.4	34.9	27.7	13.2	97.5	1.4	0	0	0	39.1	0	0	0	0
Thymol iodide	99.1	47.3	28.6	13.3	7.3	98.3	40.8	22.8	10.6	0.9	94.8	12.8	4.0	0	0
Thymol-β-D-glucoside	78.3	32.8	29.4	26.4	21.2	74.3	27.1	24.7	18.3	16.8	71.6	13.3	10.6	7.4	5.9

Thymol-monosuccinate	66.0	52.0	36.6	32.7	26.6	57.9	43.0	32.1	29.3	11.4	53.4	37.6	13.6	2.6	0.1
Tetramisole	95.3	75.1	68.4	65.1	62.7	92.9	71.0	64.3	60.7	54.1	91.6	66.4	60.9	58.3	49.3
Thiabendazole	78.5	15.4	1.8	1.5	1.1	71.1	5.6	1.0	0.7	0.3	50.1	2.7	0.5	0.3	0
Bephenium hydroxynaphthoate	97.7	84.3	82.7	73.8	69.2	94.6	70.2	55.0	51.7	46.8	85.5	56.6	9.7	3.2	1.0
1, 4-Bis (trichloromethyl) benzene	73.3	41.2	36.8	32.8	29.3	69.9	36.5	25.7	15.3	0.4	54.7	13.5	0.2	0	0
Dichlorophene	15.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2, 2′-Methylenebis (4-chloro-6-nitrophenol)	17.8	11.8	0	0	0	14.5	0	0	0	0	0	0	0	0	0
Hexachlorophene	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bithionol	0.6	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0
Bithionol-sulfoxide	38.9	6.8	5.0	4.0	2.0	1.3	0	0	0	0	0	0	0	0	0
2, 2′-Thiobis (4-chlorophenol)	0.4	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0
2, 2′-Thiobis (3, 4, 6-trichlorophenol)	0.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4, 4′-Thiobis (2, 6-dichlorophenol)	88.3	49.7	32.3	29.1	27.1	57.2	22.5	21.6	19.4	15.4	6.8	6.1	1.8	0.9	0.1
2, 2′-Thiobis (4-chloro-6-nitrophenol)	15.7	8.7	0	0	0	3.5	0	0	0	0	0	0	0	0	0
niclosamide	2.4	0	0	0	0	2.4	0	0	0	0	0	0	0	0	0
3, 5-Dibromosalicylanilide	7.3	0	0	0	0	0.4	0	0	0	0	0	0	0	0	0
3, 5, 4′-Tribromosalicylanilide	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3, 5, 4′-Trichlorosalicilanilide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3, 5, 3′ 4′-Tetrachlorosalicylanilide	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control (containing methylcellulose)	98.9	96.7	96.0	95.9	89.9	98.9	96.7	96.0	95.9	89.9	98.9	96.7	96.0	95.9	89.9
Bunamidine hydrochloride	88.0	0.9	0	0	0	48.4	0	0	0	0	0	0	0	0	0

thiazolyl)-2-imidazolidinone), bephenium hydronaphthoate (benzyldimethyl (2-phenoxyethyl)-ammonium-3-hydroxy-2-naphthoate : Alcopar, Wellcome Foundation Ltd.), hexachloroethane and 1, 4-bis (trichloromethyl) benzene (Hetol, Farbwerke Hoechst AG.). Namely, some of the drugs used were well-known anthelmintics, and the others were derivatives of compounds known as anthelmintics.

The drugs which were soluble in water were directly added into the medium in the concentrations of 10, 40 or 100 γ per ml. The drugs slightly soluble or insoluble in water were suspended or dissolved at the concentration of 2,000 γ per ml in propylene glycol. Those solutions were added to the culture medium in the same concentrations as above. To observe the effect of propylene glycol, bunamidine hydrochloride, which had shown a comparatively high scolicidal effect in the above medium, was suspended in the same concentrations in the medium containing 0.3 % methylcellulose, and the scolicidal effect of the drug in the medium without propylene glycol was compared with that in the previous medium. The media containing only propylene glycol or methylcellulose were used as controls besides the main medium.

Each of the three concentrations of each compound was tested in triplicate. The morphological changes of protoscolices and free cysts were observed in 0.5 to 1.0 ml of homogeneously mixed media at the time when the media were changed. The observations were carried out for ten days using standard and phase contrast microscopy and supravital staining with 0.2 % neutral red and 0.02 % Janus green.

RESULTS

In the preceding report, SAKAMOTO et al. (1965) described in detail the degenerative changes in the constituent cells of protoscolex and free cyst in addition to the disappearance of mobility of protoscolex and flame cell and the dropping of hooklets. These cytopathic changes in protoscolex and free cyst seem to be useful as criterion for the differentiation between living and dead protoscolices and free cysts. In the present experiment also, those morphological changes were accepted as an indication of degeneration and death of protoscolex and free cyst. The survival times of protoscolices in the media containing drugs are shown in table 1.

The decrease of the survival rate of protoscolices in the medium with drugs was conspicuous when compared with media without drugs. A significant difference in the survival rates among each group of the derivatives of the drugs was recognized.

To simplify the data, in cases where the rates of survival protoscolices on

the 2nd, 4th, 6th, 8th and 10th days of incubation in media with drugs became less than 10 per cent of the controls, the scolicidal intensities of drugs were roughly indicated by the number of plus signs; five, four, three, two and one plus signs in order of time on the 2nd, 4th, 6th, 8th and 10th days of incubation respectively. In cases where the survival rates were more than 10 per cent of the controls, these were expressed by minus signs. Then they were classified into groups consisting of derivatives from the same compound, and arranged in table 2. The data for drugs already reported by SAKAMOTO et al. (1965) was arranged for comparison with the present data.

The most rapid decrease of protoscolices survival was observed in the medium with the derivatives of salicylanilide, followed by derivatives of bisphenol. The decreased rate of protoscolices survival in the medium with bunamidine hydrochloride, a derivative of bunamidine, was also rapid. A decrease in the rate of surviving protoscolices in the media, to which derivatives of salicylanilide were added, became progressively intense in the following order: 3, 5-dibromosalicylanilide, niclosamide, 3, 5, 4′-tribromosalicylanilide, 3, 5, 4′-trichlorosalicylanilide and 3, 5, 3′ 4′-tetrachlorosalicylanilide.

The degree of the decrease of protoscolices survival in the media to which the derivatives of bisphenol were added, increased in the order as follows: 4, 4′-thiobis (2, 6-dichlorophenol), bithionol-sulfoxide, disodium-thiobis (4-chloro-6-nitrophenolate), 2, 2′-methylenebis (4-chloro-6-nitrophenol), 2, 2′-thiobis (4-chloro-6-nitrophenol), disodium-thiobis (3, 4, 6-trichlorophenolate), dichlorophen, bithionol, hexachlorophen, 2, 2′-thiobis (4-chlorophenol) and 2, 2′-thiobis (3, 4, 6-trichlorophenol).

Pyrvinium pamoate of cyanine dyes also caused a considerable decrease in the survival rate of protoscolices. The decrease in the rate of surviving protoscolices was comparatively conspicuous in the media containing hexachloroethane or thiabendazole.

The relation between a chemical structure and the scolicidal effects was investigated in the salicylanilide- and bisphenol-derivatives which gave the conspicuous decrease of surviving protoscolices. 3, 5, 3′, 4′-Tetrachlorosalicylanilide and 3, 5, 4′-trichlorosalicylanilide, which have four and three chlorines respectively, gave the highest scolicidal effect. 3, 5-Dibromosalicylanilide with two bromines was ranked next to them. The scolicidal effect of salicylanilide derivatives, accordingly, showed a tendency to increase with the number of halogen atoms, chlorines and bromines, combined with the compounds. In the salicylanilide derivatives with the same number of halogen atoms, the effect of the compounds with chlorines appeared to be more intense than that of compounds with bromines. As for the derivatives of diphenyl sulfide of bisphenol, 2, 2′-

TABLE 2 *Scolicidal effect of drugs in vitro*

CLASSIFIED	DRUG	DOSE (γ/ml)		
		10	40	100
Piperazine derivative	Piperazine adipate	−	+	##
〃	Diethylcarbamazine citrate	−	−	−
Aminoquinoline derivative	Chloroquine diphosphate	−	+	+
Phthalic acid derivative	Phthalofyne	−	−	−
Pyridine derivative	Methyridine	−	−	−
Arecoline derivative	Arecoline hydrobromide	−	−	−
Bephenium derivative	Bephenium hydroxynaphthoate	−	−	###
Benzen derivative	1, 4-Bis (trichloromethyl) benzene	−	+	##
Chlorinated hydrocarbon	Hexachloroethane	−	###	###
Antimonial	Sodium antimonyl tartarate	###	###	###
〃	Antimony pyrocatechol sodium disulfonate	##	###	###
〃	Sodium antimony gluconate	−	−	##
Arsenical	Acetarsone	−	−	−
Copper compound	*dl*-Cupric methionate	−	+	##
Phenol derivative	Disophenol	−	++	###
〃	Thymol	−	++	###
〃	Chlorothymol	−	###	###
〃	Thymol iodide	+	+	##
〃	Thymol-β-D-glucoside	−	−	++
〃	Thymol monosuccinate	−	−	++
Aminothiazol derivative	Tetramisole	−	−	+
Nitrothiazol derivative	Niridazole	++	###	###

Benzothiazol derivative	Thiabendazole	###	###	###
Cyanine dye	Dithiazanine iodide	+	###	###
〃	Pyrvinium pamoate	###	###	###
〃	Gentian violet	###	###	###
Bunamidine derivative	Bunamidine hydrochloride	###	###	###
Bisphenol derivative	Dichlorophen	###	###	###
〃	2, 2 ′-Methylenebis (4-chloro-6-nitrophenol)	###	###	###
〃	Hexachlorophene	###	###	###
〃	Bithionol	###	###	###
〃	Bithionol-sulfoxide	###	###	###
〃	2, 2′-Thiobis (4-chlorophenol)	###	###	###
〃	2, 2′-Thiobis (3, 4, 6-trichlorophenol)	###	###	###
〃	2, 2′-Thiobis (4-chloro-6-nitrophenol)	###	###	###
〃	4, 4′-Thiobis (2, 6-dichlorophenol)	−	−	###
〃	Disodium-thiobis (3, 4, 6-trichlorophenolate)	###	###	###
〃	Disodium-thiobis (4-chloro-6-nitrophenolate)	###	###	###
Salicylanilide Derivative	niclosamide	###	###	###
〃	3, 5-Dibromosalicylanilide	###	###	###
〃	3, 5, 4′-Tribromosalicylanilide	###	###	###
〃	3, 5, 4′-Trichlorosalicylanilide	###	###	###
〃	3, 5, 3′, 4′-Tetrachlorosalicylanilide	###	###	###

thiobis (3, 4, 6-trichlorophenol) with six chlorines showed a more intense effect than 2, 2′-thiobis (4, 6-dichlorophenol) with four chlorines. By comparison among hexachlorophen, 2, 2′-methylenebis (3, 4, 6-trichlorophenol), dichlorophen and 2, 2′-methylenebis (4-chlorophenol), the scolicidal effects of the first two with six chlorines were conspicuously higher than those of the last two, which have two chlorines.

In the compounds having chlorines at the same positions in the chemical structure, the scolicidal effects of 2, 2′-thiobis (4-chlorophenol) and 2, 2′-thiobis-(3, 4, 6-trichlorophenol) of diphenyl sulfide-derivatives were more potent than that of dichlorophen and hexachlorophen of diphenylmethane derivatives. 2, 2′- Thiobis (4-chloro-6-nitrophenol) of diphenyl sulfide and 2, 2′-methylenebis (4-chloro-6-nitrophenol) of diphenylmethane nitrated in the sixth position of the phenol ring were much weaker in the scolicidal effect than 2, 2′-thiobis (4-chlorophenol) and dichlorophen, which had not been nitrated in the same position. 2, 2′-Thiobis-(3, 4, 6-trichlorophenol) gave the greatest effect in the derivatives of diphenyl sulfide, but 2, 2′-thiobis (4-chloro-6-nitrophenol) showed comparatively little effect. Disodium-thiobis (3, 4, 6-trichlorophenolate) and disodium-thiobis (4-chloro-6-nitrophenolate) which were made water-soluble by the substitution of sodium revealed the weaker effect than the two original compounds with hydroxyl group as above. 2, 2′-Sulfinylbis (4, 6-dichlorophenol), which was substituted with sulfoxide for sulfonic bond of bithionol, showed a conspicuous decrease in the scolicidal effect. The scolicidal action of 4, 4′-thiobis (2, 6-dichlorophenol), in which the position of sulfide bond in the phenol ring was different from that of bithionol, was much weaker than that of bithionol. Sodium antimony gluconate with pentavalent antimony gave a lower scolicidal action than that of the trivalent antimony compounds such as antimony pyrocatechol sodium disulfonate (Fuadin, Bayer Pharmaceutical Co., Ltd.) and sodium antimony tartrate (Stibnal, Banyu-Seiyaku Co., Ltd.) which were described in the preceding report (SAKAMOTO et al., 1965).

Generally speaking, the scolicidal effect of derivatives of thymol was lower. Thymol monosuccinate and thymol-β-D-glucoside gave a comparatively higher effect. The derivatives of thymol with halogen atoms were ranked next. Namely, thymol iodide of those revealed considerable effectiveness, followed by chlorothymol.

Gentian violet of cyanine dyes showed a conspicuously higher effect, and pyrvinium pamoate also was considerably higher.

The scolicidal action of compounds known as nematocidal drugs were generally lower. However, niridazol of nitrothiazole derivatives and thiabendazole of benzothiazole derivatives gave notable effects.

The scolicidal effect of bunamidine hydrochloride suspended in the medium containing 0.3% methylcellulose was lower than that of drugs in the medium with propylene glycol.

The decrease of surviving free cysts incubated in the medium containing drugs, in other words the intensity of destructible action of drugs on the cells forming cyst wall, was approximately in parallel with that of the protoscolices mentioned above.

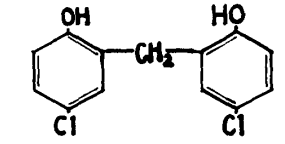
Discussion

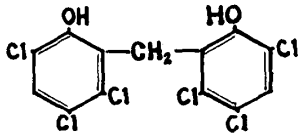
The present experiment dealt with the direct anthelmintic action of drugs against larval *Echinococcus* on the assumption that the drugs could contact the larval tissue. Some reports regarding the in vitro effects of drugs have been reported by Clunies Ross (1927), Schwabe et al. (1963), Meymerian et al. (1963), Lamy (1963, 1965), Heslop (1967), Panaitesco (1968) and Frayha et al. (1971) upon *Echinococcus granulosus*, and Sakamoto et al. (1965) and Lukashenko et al. (1970, 1971) upon *E. multilocularis*. The most important problem in the assessment for scolicidal action of drugs in the in vitro screening test is the criterion between protoscolex survival and death. Clunies Ross (1927) used, as a criterion, the lethal time of protoscolex demonstrable by the loss of clarity of the various structures, and by the cessation of active contractile movement. Schwabe et al. (1963) and Lukashenko et al. (1970, 1971) represented the activity of protoscolex by the respiratory rate. Meymerian et al. (1963) and Frayha et al. (1971) determined viability of protoscolices by their mobility, staining properties and intraperitoneal infectivity for young albino mice. Sakamoto et al. (1965) described the survival rate assessed by the cytopathic changes of constituent cells of protoscolex and daughter cyst. The object of the in vitro screening test was to assess the direct anthelmintic action of drugs against parasites. In the present experiment, the survival rate of protoscolices and free cysts was assessed on the basis of the cytopathological examination using standard and phase contrast microscopes and by supravital staining similar to the preceding report.

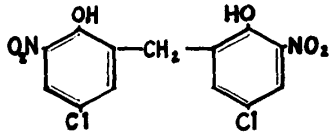
Comparing the scolicidal effects of the drugs in the present examination, salicylanilide derivatives produced the strongest effect, followed by bisphenol derivatives which included diphenyl sulfide and diphenylmethane derivatives. Bunamidine hydrochloride, cyanine dyes and antimony compounds gave a comparatively stronger effect.

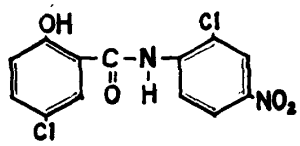
In the relation between the molecular structure and the scolicidal action of drugs, the intensity of scolicidal action increased with the increase of halogen atoms, such as chlorine and bromine, in derivatives of salicylanilide. Among the compounds with the same number of halogen atoms, the scolicidal action

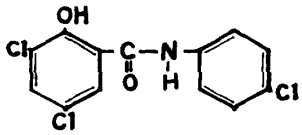
FIGURE *Derivatives of diphenylmethane, diphenyl sulfide and salicylanilide tested*

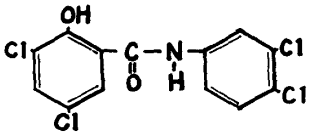

dichlorophen

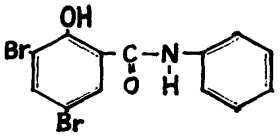

hexachlorophen

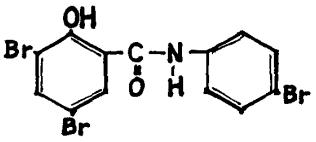

2,2'-methylenbis (4,chloro-6,nitrophenol)


niclosamide


3,5,4'-trchlorosalicylanilide


3,5,3',4'-tetrachlorosalicylanilide


3,5-dibromosalicylanilide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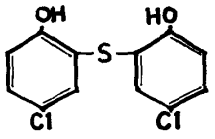
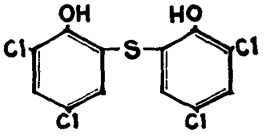
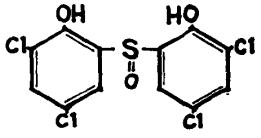
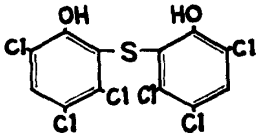
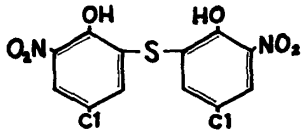
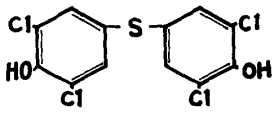

2,2'-thiobis(4-chlorophenol)

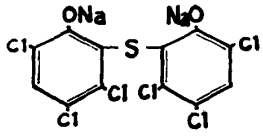

bithionol

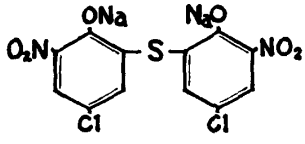

bithionol-sulfoxide


2,2'-thiobis (3,4,6-trichlorophenol)


2,2'-thiobis (4-chloro-6-nitrophenol)


4,4'-thiobis(2,6-dichlorophenol)


disodium-thiobis (3,4,6-trichlorophenol)


disodium-thiobis (4-chloro-6-nitrophenolate)

of chlorinated salicylanilide was stronger than that of brominated one. These findings bear a close resemblance to the generally well-known fact that the toxic intensity of halogenated hydrocarbons increases with the number of chlorine atoms in them. SAITO et al. (1963) tested in vitro the anthelmintic action of many derivatives of salicylanilide against *Fasciola hepatica*. The anthelmintic action of the compounds which had halogens in their salicylic base, was stronger than that of the compounds with halogen atoms on their aniline base, but the number of halogen atoms in the compounds did not have as much influence on intensity of anthelmintic action. The present results are not in full accord with their results concerning the number of halogen atoms in the compounds and intensity of the anthelmintic action. TANIGUCHI et al. (1965) examined the anthelmintic effect of four derivatives of halogenized salicylanilide and two other drugs upon liver fluke in vitro. 3, 5, 3′, 4′-Tetrachlorosalicylanilide and mixture of 3, 5, 4′-tribromosalicylanilide and 3, 5-dibromosalicylanilide showed the highest anthelmintic action. The decrease of efficiency was recognized in the order of 3, 5′-dichlorosalicylanilide, bithionol and hexachloroethane. Their findings are in accord with the present results.

The relationship between the number of halogen atoms of the compounds and the intensity of their anthelmintic action was also recognized with the bisphenol derivatives. The intensity of scolicidal action of these compounds rose with the increasing number of chlorine atoms. The diphenyl sulfide derivative and the diphenylmethane derivative which have nitro-group in the 6th position of their phenol ring showed a decline in the scolicidal action compared with the compounds without the group. The diphenyl sulfide derivatives, in which the hydrogen of the hydroxide group was replaced by sodium, and bithionol sulfoxide, in which the sulfoxide-bond replaced the sulfide-bond of bithionol, revealed a decline in the scolicidal action. The scolicidal action of the compound, in which the position of sulfide was moved from the 2, 2′ of the phenol ring to the 4, 4′ position, had shown an extreme decline. On the other hand, MURAKOSHI et al. (1969) demonstrated that bithionol inhibited the action of fumarate reductase in carbohydrate metabolism, and that this inhibitive action was due to the hydroxide group. From this finding, they conjectured that the halogen in the phenol ring accelerated the dissociation of hydroxide groups in those compounds. From the fact that most of organic compounds with halogen atoms show anthelmintic action, they considered that the halogens in the compound have a pharmacological significance for anthelmintic action. Their opinion seems to apply in the relationship between halogens and anthelmintic action of the compounds in the present study.

YOKOGAWA et al. (1961) recognized strong vermicidal action of bithionol against

excysted larva of *Paragonimus westermani* and *P. ohirai*. HOSAKA et al. (1967) reported that serum of dogs which were given 300 mg per kg of bithionol sulfoxide showed a vermicidal effect against *Clonorchis sinensis* and *P. westermani*. HIGAKI et al. (1963) reported that the highest concentration of bithionol and bithionol sulfoxide was observed in the serum of rabbits 4 hours after oral doses of 100 and 200 mg per kg of these drugs, and that the concentrations of bithionol in the serum of rabbits which were given 100 and 200 mg per kg, were about four times and eight times of that of bithionol sulfoxide. HARA & SUZUI (1967) reported that about 200 γ per ml of bis (2-hydroxy-3-nitro-5-chlorophenol) sulfide was observed in the bovine serum 6 to 12 hours after oral medication of 100 mg per kg of the drug. MURAKOSHI et al. (1964) described that bithionol uncoupled oxidation at the low concentration of 10^{-8} M, but that it inhibited anaerobic and aerobic respiration of *Paragonimus westermani* and *Fasciola hepatica* in concentration of 10^{-3} to 10^{-4} M. They also stated that the concentrations of bithionol in the liver and kidney were about ten times of that in the blood. When the concentration of bisphenol derivatives in the blood and liver as mentioned in the above reports, and scolicidal action of the derivatives in low concentration which were observed in the present experiment are considered, it is conjectured that those drugs are hopeful for pharmacotherapy of echinococcosis.

Sodium antimony gluconate, pentavalent antimonial gave a lower scolicidal action than that of the two trivalent antimonials, sodium antimony tartrate and antimony pyrocatechol sodium disulfonate, described in the preceding report. YAMAGUTI et al. (1962) stated that these trivalent antimonials showed quite a strong effect in the screening test for *Clonorchis sinensis* in vitro. MANSOUR & BUEDING (1954) and BUEDING & MANSOUR (1957) described that the trivalent antimonials, stibophen (antimony pyrocatechol sodium disulfonate) and antimony potassium tartrate (tartar emetic) selectively inhibited schistosome phosphofructokinase without affecting the host enzyme. This action of the trivalent antimonials seems to be due to their greater solubility when compared with pentavalent antimonial. SCHWABE et al. (1963) reported that one of the trivalent antimonials, potassium antimony tartrate markedly inhibited protoscolex respiration of *E. granulosus*; on the other hand another trivalent antimonial (stibophen) was without an appreciable effect. LUKASHENKO et al. (1970), however, stated that potassium antimony tartrate permeated readily through the cyst wall of *E. multilocularis* in vitro but only slowly acted on the protoscolices. LUBINSKY (1970) observed that antimony dimercaptosuccinate, trivalent antimonial, did not inhibit the growth of multilocular echinococcal cysts in mice. The author has not explanatory data as to the cause of this disagreement among the results with trivalent antimonials in the various experiments mentioned above, excepting

the difference of the species of parasites used.

As to the effect of cyanine dyes in vitro, SCHWABE et al. (1963) and LUKASHENKO et al. (1970, 1971) reported that gentian violet markedly inhibited the respiratory activity of protoscolices of *E. granulosus* and *E. multilocularis*, respectively. SAKAMOTO et al. (1965) described the remarkable parasitocidal action of dithiazanine iodide upon larval *E. multilocularis* incubated in vitro in the preceding report. In the present experiment, gentian violet showed a stronger action than that of dithiazanine iodide, and the action of pyrvinium pamoate held the intermediate rank between them.

Bunamidine hydrochloride gave a remarkable scolicidal action. Many investigators[2,7,12,14,21,47,53,61] reported the remarkable anthelmintic effect of the derivatives of bunamidine upon *E. granulosus*. SAKAMOTO et al. (1971) recognized that the drug also showed a high anthelmintic action against adult *E. multilocularis*. The drug is, however, stimulative for mucous membranes of the host. Before using the drug for therapy of larval echinococcosis in the liver and lung, there are many problems which should be resolved.

HANSTEIN (1957) reported that palmitic acid thymol ester resembling the oily solution of thymol which had been already stated by CUERVO GARCÍA (1951), THIODET (1955) and TOCCO (1955) to be effective upon human echinococcosis gave a therapeutic effect against multilocular echinococcosis. YAMADA et al. (1962) and YAMADA (1962, 1963) also reported the same result as that described above. However, the present in vitro test revealed that the scolicidal intensity of thymol and its derivatives was extremely weak. On the other hand, administered thymol has been thought to conjugate with sulfuric acid or glucuronic acid or to change into thymoquinone in the liver of the host. AKAGI (1962) considered that the conjugated thymol or thymoquinone derived from thymol acted as anthelmintics. To confirm their opinion, the present author injected thymoquinone into mice experimentally infected with *E. multilocularis*. No evidence of therapeutic effect was recognized in the microscopical examination of echinococcal tissue in the liver of the mice. YAMASHITA (1961) and YAMASHITA et al. (1962) reported that palmitic acid thymol ester produced a tendency to accelerate the development of larval echinococcal tissue of mice rather than inhibit it, and that the greater part of the oily solution injected remained unabsorbed from the region of intramuscular injection. The same findings were also submitted to the Hokkaido Health Department by a group of investigators consisting of pharmacologists and pathologists. They had been supported by a subsidy from the Hokkaido Prefectural Office for the pharmacological study on the effect of thymol upon echinococcosis. From the above facts, the effect of thymol upon human echinococcosis is considered to be doubtful.

The scolicidal action of compounds well-known as nematocidal drugs were generally lower. However, thiabendazole of bendathiazole derivative, effective for various adult and larval nematoda, gave a notable effect in the present experiment. BEAL & MARKELL (1966) and LUBINSKY (1970) stated that thiabendazole was ineffective as a therapy for multilocular echinococcosis in cotton rats and mice, respectively. Niridazole of nitrothiazole derivative gave comparatively strong effect in the present test. PELLEGRINO et al. (1962) and LAMBERT (1964) reported that the drug showed schistosomicidal activity.

In the present experiment, three kinds of media were used. The first was the main medium, to which calf serum was added at the concentration of 20 per cent to medium 199; the second 0.5, 2 and 5% solution of propylene glycol in the medium; and the third contained 0.3% solution of methylcellulose in the medium. Most of anthelmintics for intestinal cestodes manifest low toxicity for the host, because they are insoluble in water and would hardly be absorbed through the mucous membrane of the intestine of the host.

In the present experiment, bunamidine hydrochloride, which was added as the solution in propylene glycol into the medium, manifested a stronger scolicidal effect than that suspended in the medium with methylcellulose. This finding is thought to show that the absorption of the drug by protoscolex was assisted by presence of propylene glycol.

Niclosamide, which was added into the medium together with propylene glycol, gave a strong scolicidal effect in the present test. LAMY (1965) observed that the suspension of niclosamide at the concentration of 1: 1,000,000 to 1: 10,000,000 in water with a trace of gum arabic destroyed the protoscolices of *E. granulosus* in vitro. STRUFE & GÖNNERT (1967) investigated the influence of Atebrine, dichlorophen and niclosamide on the metabolism of *Hymenolepis diminuta*. Of these drugs, niclosamide affected most strongly the process of energy production in tapeworms, and showed the most intensive stimulation of anerobic glycolysis. SAKAMOTO et al. (1971) found adult *E. multilocularis* remaining in the intestine of the infected dogs which were given 500 and 700 mg per kg of niclosamide. KURELEC & RIJAVEC (1961), DELAK et al. (1963, 1965), CORDERO DEL CAMPILLO et al. (1965), CACHO LÓPEZ et al. (1963), FORBES (1963) and MERDIVENCI (1968) treated with niclosamide dogs infected with *E. granulosus*. They reported that the anthelmintic effect of the drugs was not so strong considering the high dosage of the drug. Therefore, if the drug is administered together with an additive such as propylene glycol, the drug would give an increase in the intensity of anthelmintic action and toxicity. Accordingly, the present author considers it to be significant that an additive such as propylene glycol should be examined clinically together with anthelmintics in the future.

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