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STUDIES ON ECHINOCOCCOSIS XIX
HEAT STABLE ANTIGEN IN CYSTIC FLUID OF
ECHINOCOCCUS MULTILOCULARIS

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INTRODUCTION

In the serological diagnosis of hydatid disease, especially the hemagglutination test, antigen prepared from hydatid fluid is superior to that of other hydatid tissue. The fluid, however, contains a significant amount of host components and is thought to show nonspecific serological reactions to sera from patients afflicted with diseases other than echinococcosis (KAGAN et al., 1960⁸). KAGAN and his coworkers have attempted fractionation works, but they have failed to obtain pure an antigenic substance without host components (KAGAN et al., 1960⁷); KAGAN, 1963 and NORMAN et al., 1966).

In the serological diagnosis of unilocular echinococcosis, BOYDEN's indirect hemagglutination test was found to be superior to the complement fixation test in sensitivity at the level of employing crude antigen (KAGAN et al., 1959). As for diagnosis of multilocular echinococcosis, satisfactory results could not be obtained in experiments when cystic fluid from experimental animals was used as the antigen in the hemagglutination test (KAGAN et al., 1960⁷).

In this study, the complement fixation test (CF test) was employed in the titration of the antigen substance and the antiserum. The author also carried out the agar gel diffusion techniques for comparisons of raw and heated antigen substance in test with sera from infected and immunized animals. For inactivation of antigenicity of host components in the cystic fluid of *E. multilocularis*, heat treatment experiments were conducted.

MATERIALS AND METHODS

Source of antigen The liver foci obtained from five CF #1 mice (♀♀) were used for starting materials of antigen. The mice were inoculated with 200~300 eggs of the Alaskan strain of *Echinococcus multilocularis* 10 months ago when they were 60 days old. The liver foci were homogenized gently with a waring blender keeping the scolices safe from destruction and were centrifuged at 10,000 rpm for 30 minutes. The supernatant was stored at -20°C for three months, then the melted supernatant was used in the study after

discarding the centrifuged precipitates.

Gel filtration Sephadex G 75 was used through the experiment. The fluents were submitted to analyse for protein, carbohydrate and antigen titer. Protein was determined spectrophotometrically at 280 m μ . Carbohydrate was determined by Anthrone method. Antigen titer was determined by CF test.

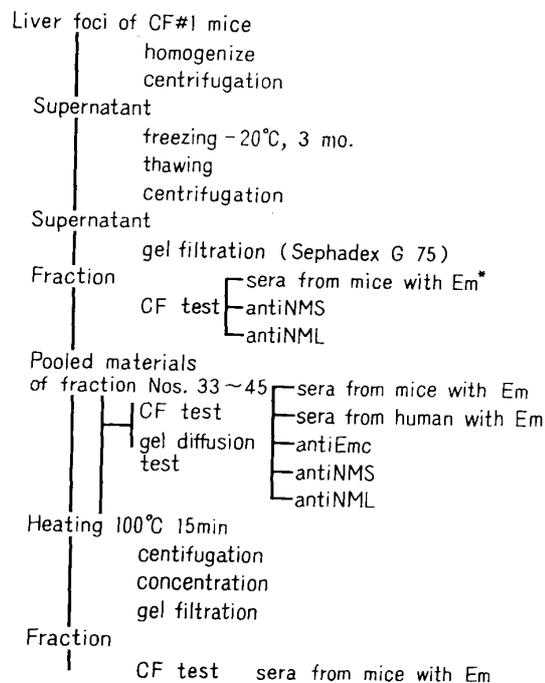
Heat stable antigen Heat stable antigen was prepared through the heating at 100°C for 15 minutes.

Antisera preparation The cystic fluid of *E. multilocularis* (Emc), normal mice sera (NMS) and livers (NML), were used as the antigenic material. The antisera to Emc (antiEmc), to NMS (antiNMS) and to NML (antiNML) were prepared from rabbits. Emc was obtained by the same procedure described above and was mixed with an equal amount of FREUND's complete and incomplete adjuvant (Difco) respectively. Four rabbits were inoculated subcutaneously once every 10 days for 40 days, with 5 ml of antigen mixed with adjuvant. The first and second injections were antigen mixed with complete adjuvant and the third and fourth injections were antigen with incomplete adjuvant. Thirty minutes before the intravenous booster injection of 5 ml of antigen was carried out, 10 days after the final inoculation, the subcutaneous inoculation of antigen (5 ml) was performed for protection against shock. After a rest period of 7 days or more, each rabbit was sacrificed for bleeding. AntiNMS and antiNML were prepared by the same procedure as antiEmc. With regard to normal mice livers, they were collected from 20 mice, homogenized with a waring blender and a teflon-glass tissue grinder in an equal amount of 0.9% NaCl solution with merthiolate (1 : 10,000).

Complement fixation test The performance of this test was carried out by KOLMER's modification. The hemolytic system used, was 0.2 ml of 3 units of hemolysin, an equal amount of 3% sheep red corpuscles and 0.4 ml of 2 units of complement. To mixtures of 0.2 ml of two fold serial dilution of antigens and 0.2 ml of 4 units of antisera, 0.4 ml of 2 units of complement was added and held in cold storage for 9~12 hours. They were exposed to room temperature for 15 minutes, then mixed with sensitized red corpuscles. They were incubated in a water bath at 38°C for 30 minutes. The result was judged as positive at the point where inhibition of the hemolysis of sheep red corpuscles was 75% or more.

Agar gel diffusion test The micro-slide technique was used. One percent agar (special agar, Difco) in 0.9% NaCl solution with merthiolate 1 : 10,000 were used for the gel plate. Glass slides 50×25 mm were prepared and 4 ml of melted agar was pipetted on the slide. After the agar solidified, 4 peripheral wells of 3 mm diameter were cut in a rhomb about a center well. The distance between the outer rings of the well and the center well was 5 mm. After each antigen and each serum was delivered to each well, the slide was incubated in a moist chamber at 37°C for 24 hours, and the same antigen or serum was pipetted into the well again. After a week of incubation, photographs were taken using reflecting light. The precipitates in the agar gel were stained with periodic acid-Schiff (PAS) for carbohydrates. Oxidation by periodic acid was carried out for only a few seconds unless the agar gel was stained. In the gel diffusion study, the reactants used are shown in the table.

FIGURE 1 Schema for fractionation, CF test and gel diffusion test



* Em: *Echinococcus multilocularis*

The schema for fractionation, CF test and gel diffusion test is shown in figure 1.

EXPERIMENTALS AND RESULTS

The results of the fractionation experiment using Sephadex G 75 column are shown in figure 2.

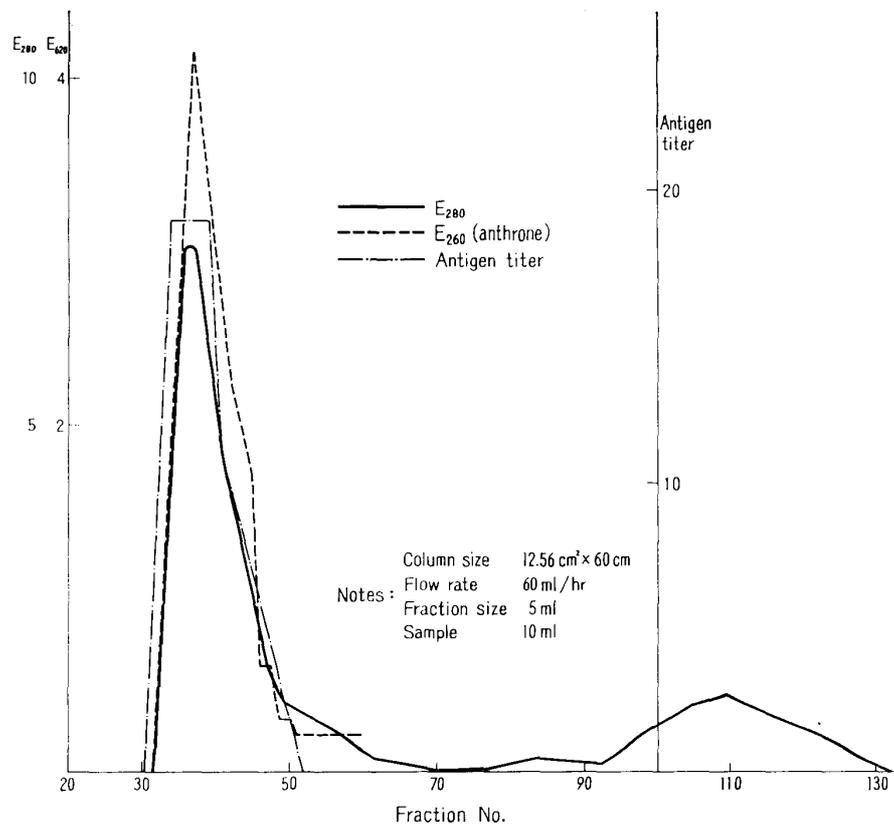
In fractions Nos. 32~45, precipitates were formed. Each fraction was centrifuged to discard precipitates, then spectrophotometric density was measured.

For determination of the serum titer to be used in the test proper, box titration was carried out on the crude Emc and sera from infected mice (8 months post infection). In the test proper, to each 0.2 ml of two fold serial dilution of each Emc fractionated, 0.2 ml of 4 units of the sera were mixed, and the CF test was carried out. After the antigen titer of each fraction was determined, titer per E₂₈₀ was calculated. The titer per E₂₈₀ of each fraction is shown in figure 2.

In figure 2, materials showing high titer were contained in fractions Nos. 33~45. Pooled fractions of Nos. 50~70 contained colored materials and preliminary data in the gel diffusion test showed that they were components of host origin.

Moreover, the antigen titer per E₂₈₀ in fractions Nos. 38, 43 and 56 in test with antiNMS was respectively 75~150, 125~250 and 180 in the CF test, also in the test with antiNML, the antigen titer was respectively 75~150, 600 and 360.

In fractionation by gel filtration of Sephadex G 75, fractions Nos. 33~45 showed a high

FIGURE 2 *Chromatogram of crude Emc*

antigen titer to the sera from infected mice, but contained host components. The fraction also showed a high titer to antiNMS and antiNML. Fractions Nos. 33~45 were pooled and submitted to heating experiments. Then comparative studies of raw and heated fractionated Emc were carried out using the CF test (box titration) with the following antisera. The sera used were those from infected mice (8 months post infection), antiEmc (absorbed with NMS & NML), antiNMS and antiNML. The results obtained are shown in figures 3, 4, 5 and 6.

Another lot of crude Emc, raw and heated, used for the test was pooled with human multilocular echinococcosis sera, the result of that experiment is also shown in figure 7.

As seen in figures 5 and 6, raw fractionated Emc showed a high antigen titer, but on the contrary, heated fractionated Emc in test with the same sera showed no titer even in dilution of 1 : 10 of the sera tested. Also, heated fractionated Emc in dilution of 1 : 5 and 1 : 10 was only reactive to the antiNML at a dilution of 1 : 10. However, in the test with sera from infected mice and human beings, antigenicity was present in heated Emc, but decreases in the antigen and serum titer were recognized (figs. 3 & 7). In figure 4, the serum titer in antiEmc (absorbed by NMS & NML) was not so low in comparison with data shown in figures 3 and 7. In this experiment, absorbed antiEmc was used. The procedure of absorption was as follows: AntiEmc 7 vol., NMS 1 vol. and NML 0.5 vol.

FIGURE 3 Comparisons between raw and heated fractionated Emc in CF test with sera from [mice with *E. multilocularis*

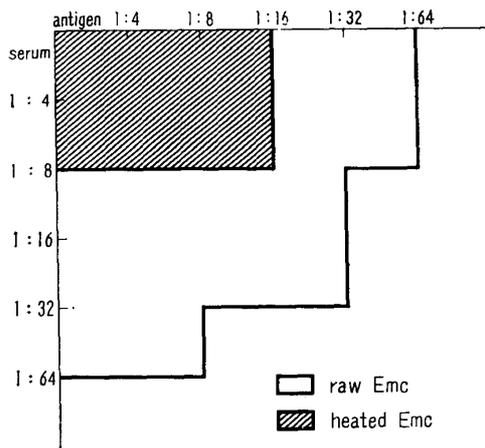


FIGURE 4 Comparisons between raw and heated fractionated Emc in CF test with antiEmc (absorbed by NMS & NML)

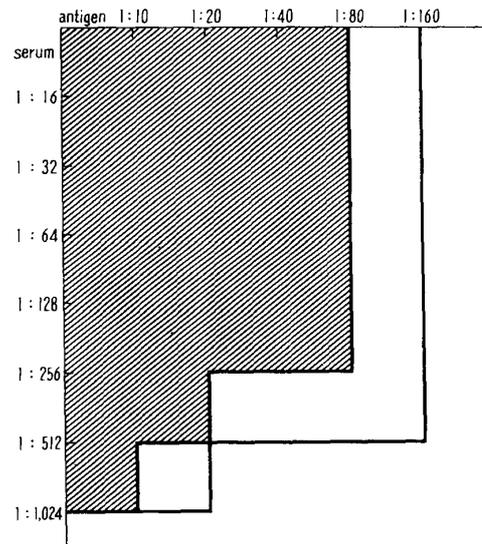
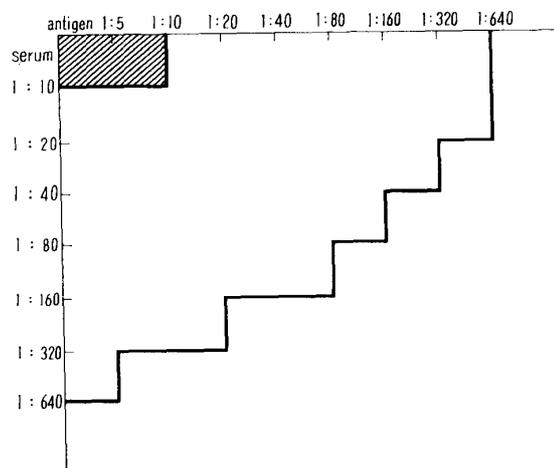


FIGURE 5 Comparisons between raw and heated fractionated Emc in CF test with antiNMS



were mixed, then incubated at 37°C for 2 hours, and stored at 4°C for 12 hours. Precipitates were discarded by centrifuge, and the supernatant was used for the CF test. For clarifying the degree of absorption, the serum was placed on a gel diffusion plate between raw and heated Emc, NMS and NML (fig. 24).

The second gel filtration of Sephadex G 75 was carried out using the heated and concentrated materials (1/10 vol.) of the pooled fractions Nos. 33~45. Chromatogram is shown in figure 8.

FIGURE 6 Comparisons between raw and heated fractionated *Emc* in CF test with antiNML

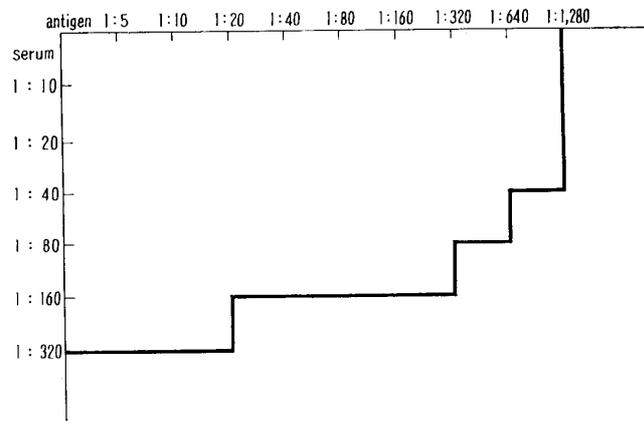


FIGURE 7 Comparisons between raw and heated *Emc* in CF test with sera from human with *E. multilocularis*

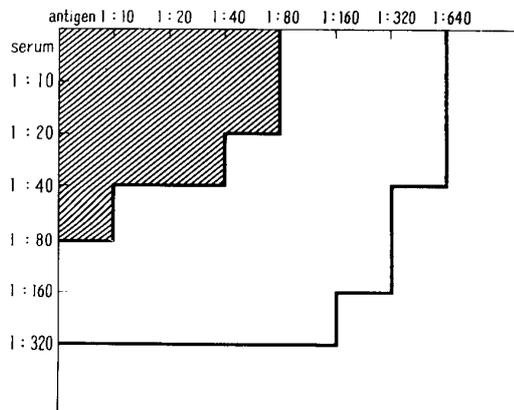
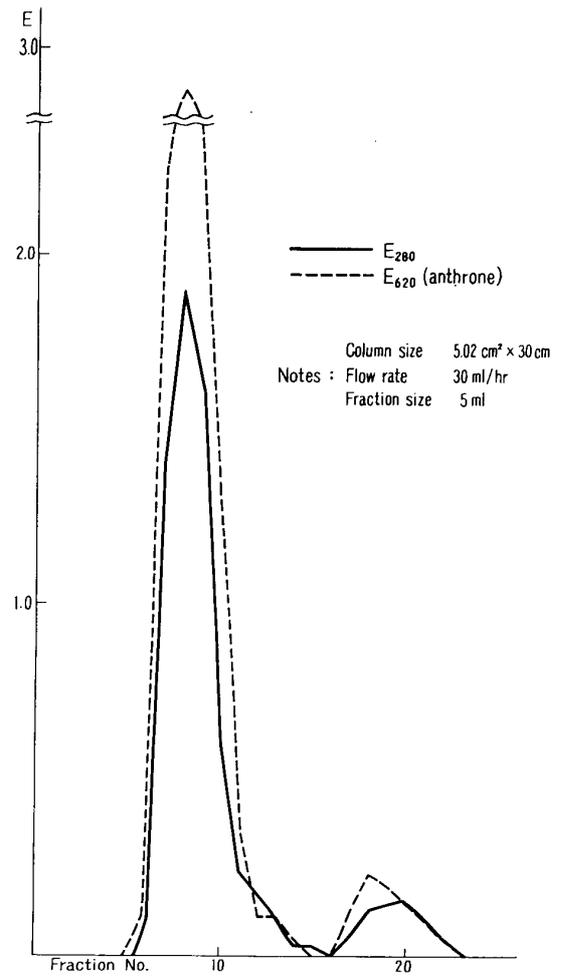


FIGURE 8 Chromatogram of heated *Emc*



Reactive materials were contained within fractions Nos. 7~10. The antigen titer of fractions Nos. 7~10 was 1:8, 1:16, 1:16 and 1:8 respectively in test with sera from the infected mice. The ultraviolet absorption spectrum of the reactive fractions showed that there was no maximum peak in wave length from 230~300 m μ .

Gel diffusion test In the study, the antigens used were raw and heated fractionated Emc. The former was concentrated 1/8 volume with polyethylene glycol, and the latter was the same materials as used for the second gel filtration. The reactants and their arrangement are shown in the table.

Comparisons of raw and heated Emc in test with sera from infected mice (figs. 9 & 10)

As shown in figures 9 and 10, the precipitates formed were almost similar both in raw and heated Emc in test with sera from infected mice. But in figure 10, agar well No. 4, the serum from mouse 120 days post infection case No. 5, a precipitate was formed on the outermost side of the raw Emc. When the precipitates were stained with PAS, the outermost precipitate was stained slightly, but the other main precipitates were stained very strongly.

Comparisons of raw and heated Emc in test with sera from human patients (figs. 11 & 12)

Precipitates formed between heated Emc and human sera with *E. multilocularis* were weaker than precipitates formed between raw Emc and human sera with *E. multilocularis*.

Comparisons of raw and heated Emc in test with antiEmc, antiNMS and antiNML (fig. 13)

In figure 13, common precipitates of host origin were formed on the left hand (between raw Emc & antisera), but these precipitates were not found on the right hand (heated Emc). Among the precipitates originated from the parasites which were formed between raw Emc and antisera, the outermost precipitate was not found between heated Emc and antisera. This precipitate was not often found in the test with sera from mice with *E. multilocularis* and raw Emc.

Comparisons of raw NMS and NML, and heated NMS and NML in test with homologous antisera (fig. 14)

An attempt was made to inactivate the antigenicity of NMS and NML by heat treatment. In this study, the procedure for heating and concentration was as follows: Normal mice livers (NML) were homogenized with a waring blender and a teflon-glass tissue grinder. To 1 ml of homogenates of livers, 9 ml of water was mixed and 0.5 ml of 5% sodium bicarbonate was added, and heated at 100°C for 15 minutes in a water bath. The final pH was adjusted to 7.2 by 0.1 N hydrochloric acid. After centrifugation, the supernatant was concentrated to the original volume. Heated NMS was made by the same method. As shown in figure 14, many precipitates were formed between the raw antigens and the homologous antisera, and precipitates were formed also between NMS and antiNML, and between NML and antiNMS. However when heated NMS was tested with antiNML and antiNMS, precipitates were not found between the former, and only slightly observed between the latter. When heated NML was tested with antiNML and antiNMS, precipitates were not formed.

TABLE *Reactants used in gel diffusion test*

CENTER WELL (antigen)		PERIPHERAL WELL (serum)				NOTES (fig. No.)
A	B	1	2	3	4	
fractionated Emc raw	fractionated Emc 100°C, 15 min	mouse Em 80 days, No. 1	mouse Em 80 days, No. 8	mouse Em 120 days, No. 1	mouse Em 80 days, No. 14	9
		mouse Em 120 days, No. 2	mouse Em 120 days, No. 3	mouse Em 120 days, No. 4	mouse Em 120 days, No. 5	10
		human Em No. 1	human Em No. 2	human Em No. 3	human Em No. 4	11
		pooled human Em No. 1	pooled human Em No. 2	human Em No. 5	mouse Em 120 days, No. 5	12
		antiEmc	antiNML	antiNMS	mouse Em 120 days, No. 5	13
crude Emc 56°C, 30 min	crude Emc raw	antiEmc	antiNML	antiNMS	mouse Em 10 mo.	21
crude Emc 70°C, 20 min	crude Emc 70°C, 10 min					22
crude Emc raw	crude Emc 100°C, 15 min					23*

* Absorption test

antigen Emc: cystic fluid of *Echinococcus multilocularis*
 NMS: normal mice sera
 NML: normal mice livers

antiserum mouse (human) Em: serum from mouse (human) with *Echinococcus multilocularis*
 antiEmc: rabbit serum immunized with cystic fluid of *Echinococcus multilocularis*
 antiNMS: rabbit serum immunized with normal mice sera
 antiNML: rabbit serum immunized with normal mice livers

TABLE (continued) *Reactants used in gel diffusion test*

CENTER WELL (serum)		PERIPHERAL WELL (antigen)				NOTES (fig. No.)
A	B	1	2	3	4	
antiNML	antiNMS	NMS	NMS 100°C, 15 min	NML	NML 100°C, 15 min	14
		crude Emc 70°C, 10 min	crude Emc 70°C, 20 min	crude Emc raw	crude Emc 56°C, 30 min	15
		crude Emc 100°C, 15 min	crude Emc raw	crude Emc raw (1 : 2)	crude Emc 100°C, 5 min	16
mouse Emc 10 mo.	antiEmc	crude Emc 70°C, 10 min	crude Emc 70°C, 20 min	crude Emc raw	crude Emc 56°C, 30 min	17
		crude Emc 100°C, 15 min	crude Emc raw	crude Emc raw (1 : 2)	crude Emc 100°C, 5 min	18
		crude Emc 70°C, 10 min	crude Emc 70°C, 20 min	crude Emc raw	crude Emc 56°C, 30 min	19*
antiEmc	mouse Emc 10 mo.	crude Emc 100°C, 15 min	crude Emc raw	crude Emc raw (1 : 2)	crude Emc 100°C, 5 min	20*
antiEmc (absorbed by NMS & NML)		NML	NMS	fractionated Emc raw	fractionated Emc 100°C, 15 min	24

* Absorption test

From the experimental results mentioned above, antigenic substances of host components which were contained in Emc were inactivated by heating at 100°C for 15 minutes, while antigenic titer originated from parasite decreases in some extent. Therefore, mild heat treatment was performed. The temperature and time combination was 56°C 30 minutes, 70°C 10 and 20 minutes respectively. For the control study, the results obtained in experiments of heat treatment as high as 100°C for 5 and 15 minutes will be also mentioned (figs. 15~23).

As shown in figure 15, partial inactivation of host components was observed by heating as high as 70°C for 10 and 20 minutes in test with antiNML, but many reactive elements still remained. When each antigen was tested with antiNMS, the substance which was formed the outermost precipitate was inactivated at 70°C for 20 minutes or below, but most of the reactive elements of host origin could not be inactivated. Reactive elements of host origin were completely destroyed by heating at 100°C for 5 and 15 minutes (fig. 16).

In figures 17 and 18, each Emc heated was not affected by heating when each Emc was tested with serum from an infected mouse, but on the right hand, the process of inactivation of the host's reactive elements was observed. Remarkable differences were observed in these figures, when Emc heated as high as 100°C for 5 and 15 minutes and at 70°C for 20 minutes or below in test with the antisera. In other words, antigen substances in Emc which formed the outer precipitates surrounding the center well were destroyed by heat at 100°C for 5 minutes or over.

The fact becomes more clear to see in figures 19 and 20. In these experiments, absorption technique was applied as follows. Mixtures of antiNMS and antiNML were pipetted in all wells on the agar plate. After they were absorbed, the same reactants as figures 17 and 18 were delivered.

The inactivation process caused by heat treatment of Emc becomes more evident in the following experiment (figs. 21 & 22). Host components which were contained in Emc were inactivated gradually according to the degree and length of heating. Antigens originated from parasite were affected partially by heating above 100°C for 15 minutes, but not under 70°C for 20 minutes.

DISCUSSION

This work is based on the following assumption. Host components in cystic fluid are labile to heat because electrophoretic analysis of cystic fluid is similar to the serum of the host infected with *E. multilocularis* (GOODCHILD & KAGAN, 1961). Moreover, many components of host origin in cystic fluid are identified by OUCHTERLONY plate method (KAGAN & NORMAN, 1961). The heat treatment, as high as 100°C for 15 minutes, is a very useful method for inactivation of antigen substances of host origin.

On the contrary, most of the antigenic substances originated from the parasite are shown heat stable in the gel diffusion test, although decreases in antigen titer are recognized in the CF test. The serum titer of mice and human beings with multilocular echinococcosis is also decreased in the CF test with heated Emc in comparison with raw Emc. Therefore, mild heating was carried out for main-

taining the titer of antigen originated from the parasite and at the same time inactivating antigens of the host origin. However, satisfactory results could not be obtained. Of the antigens originated from the parasite, heat labile antigens were recognized, but with this antigen, the sera from most cases of infected mice which were used in the study, were not reactive except the serum from one case 120 days post infection. It could not be confirmed in this study that the antibody reacting with the heat labile antigen was present or not in sera from human beings with *E. multilocularis*, because the precipitates formed were very slight even when raw Emc was used.

Some antigenic components in Emc are stable at 70°C for 20 minutes, but are destroyed at 100°C for 5 minutes. These substances are reactive to antiEmc but not reactive to sera from most cases of mouse, antiNMS and antiNML. Also antibodies against these substances in antiEmc were not reactive to NMS. Whether these substances originated from the parasite or host was not identified. At any rate, the value of these substances in diagnosis is doubtful, since these substances are not often reactive to serum from mice with *E. multilocularis*.

HARIRI et al. (1965) reported the effect of temperature on antigens in the cystic fluid of sheep in the hemagglutination test. The antigen was inactivated completely at 80°C for 5 minutes. They pointed out the differences of heat stability in antigens in the hemagglutination test and in the CF test reported by BACIGALUPO (1925). If a comparison of results was permitted as to the antigen in cystic fluid of *E. granulosus* and *E. multilocularis*, the author considers that the differences are dependent on the nature of the antigen. In other words, the antigen, in the hemagglutination test is protein in nature and is inactivated by heating, so the antigen cannot combine with erythrocytes treated with tannic acid, but in the CF test or gel diffusion test, antigenic substances are not always protein in nature. The author considers polysaccharides take a part as an antigen in heat stable antigenic substances, because precipitates which are formed between heat stable antigens and serum from mice with *E. multilocularis* are stained strongly with PAS. Also, anthrone positive materials are contained in reactive fractions obtained by Sephadex gel filtration in both raw and heated Emc.

SUMMARY

For inactivation of antigenicity of host components in cystic fluid of *E. multilocularis*, heat treatment experiments were conducted. Fractions which are reactive to serum from infected mice with *E. multilocularis* were collected by gel filtration of Sephadex G 75, and were heated at 100°C for 15 minutes. For a comparative study, mild heating was also performed. For serological tests, the CF test and gel diffusion test were used.

Experimental results obtained may be summarized as follows :

1) Reactive components of host origin in cystic fluid are partially lost at 70°C for 20 minutes and are almost destroyed at 100°C for 15 minutes.

2) In the gel diffusion test, most antigens originated from the parasite in cystic fluid are stable at 100°C for 15 minutes in test with serum from mice with *E. multilocularis*, and these precipitates are stained strongly with PAS. So, polysaccharides seem to take a part in these precipitates. In antigens originated from the parasite, heat labile antigens are also present while it is not often reactive in test with sera from mice.

3) In the CF test, heated fractionated Emc titer is low, in comparison with raw fractionated Emc in test with sera from human beings and infected mice.

The author wishes to express his cordial gratitude to Prof. Jiro YAMASHITA for his kind guidance. He is indebted to Dr. Hiroo IIDA, Hokkaido Institute of Public Health for supplying the sera of men infected with *E. multilocularis*.

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EXPLANATION OF PLATES

PLATE I

Figures 9~13, center well A; fractionated Emc (raw) and center well B; fractionated Emc (100°C, 15 min)

Fig. 9 Peripheral well 1; serum from CF #1 mouse, 80 days post infection, No. 1, well 2; 80 days, No. 8, well 3; 120 days, No. 1 and well 4; 80 days, No. 14

Fig. 10 Peripheral well 1; serum from CF #1 mouse, 120 days post infection, No. 2, well 2; 120 days, No. 3, well 3; 120 days, No. 4 and well 4; 120 days No. 5

Fig. 11 Peripheral well 1~4; serum from human with *E. multilocularis* Nos. 1~4

Fig. 12 Peripheral wells 1 & 2; pooled sera from human with *E. multilocularis*, Nos. 1 & 2, well 3; serum from human with *E. multilocularis*, No. 5 and well 4; serum from CF #1 mouse, 120 days No. 5

Fig. 13 Peripheral well 1; antiEmc, well 2; antiNML, well 3; antiNMS and well 4; serum from CF #1 mouse 120 days, No. 5

Figures 14~16, center well A; antiNML and center well B; antiNMS

Fig. 14 Peripheral well 1; NMS, well 2; NMS (100°C, 15 min), well 3; NML and well 4; NML (100°C, 15 min)

Fig. 15 Peripheral well 1; crude Emc (70°C, 10 min), well 2; crude Emc (70°C, 20 min), well 3; crude Emc (raw) and well 4; crude Emc (56°C, 30 min)

Fig. 16 Peripheral well 1; crude Emc (100°C, 15 min), well 2; crude Emc (raw), well 3; crude Emc (raw 1:2) and well 4; crude Emc (100°C, 5 min)

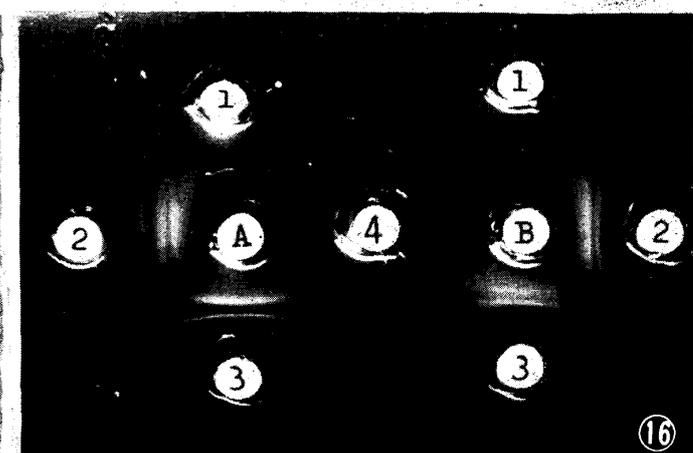
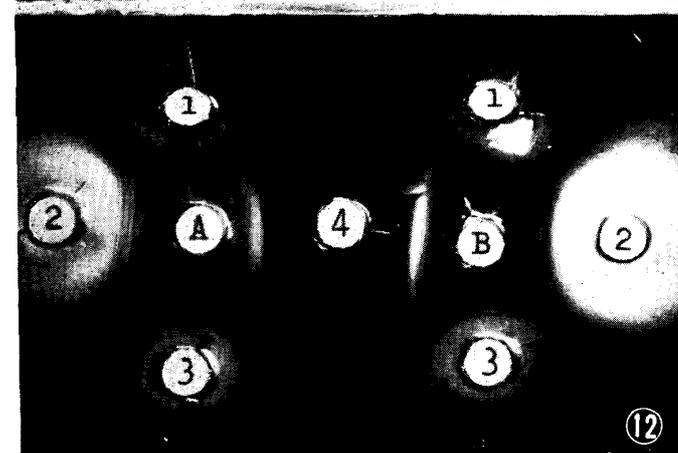
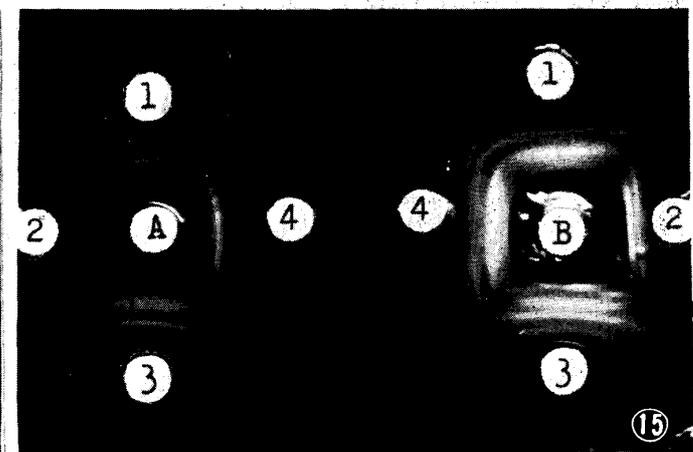
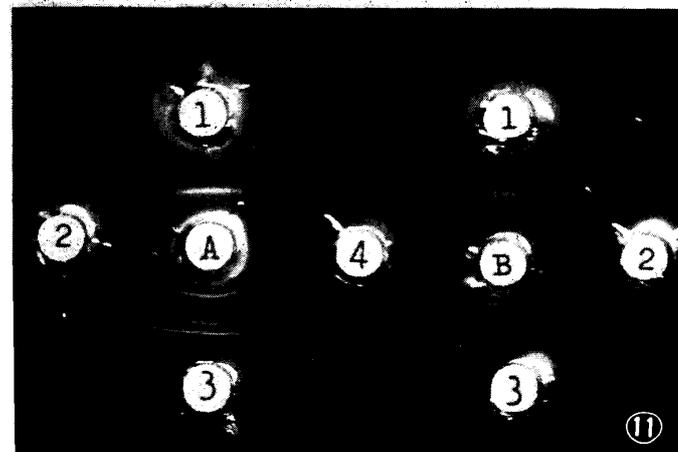
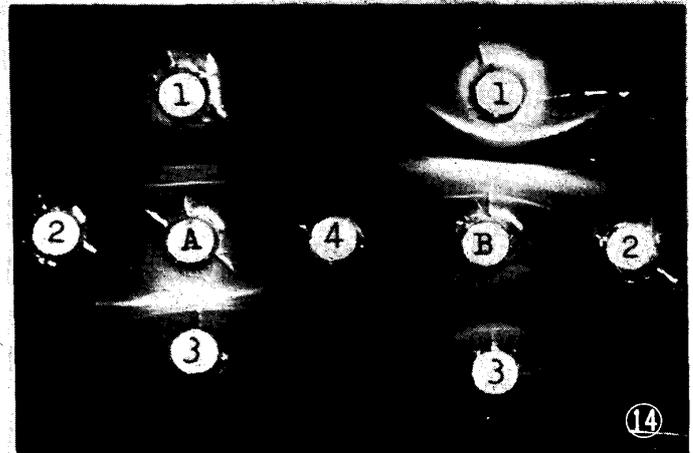
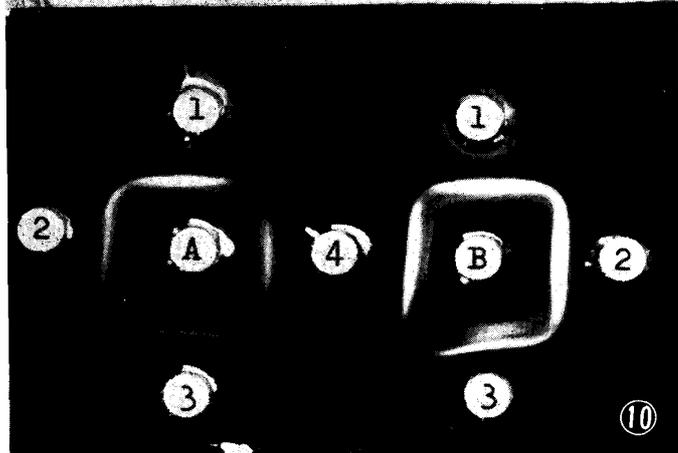
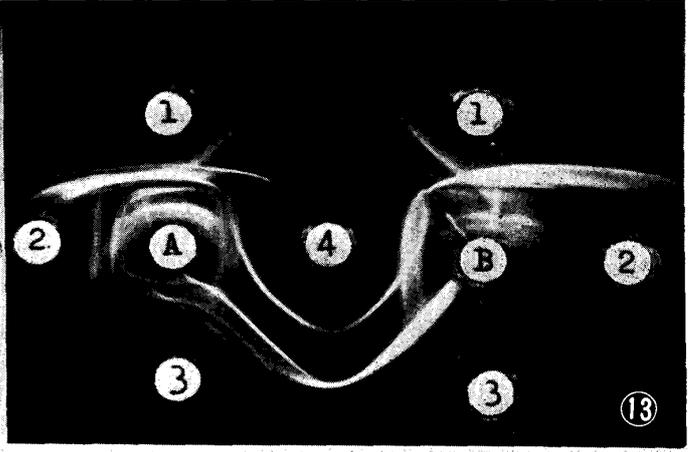
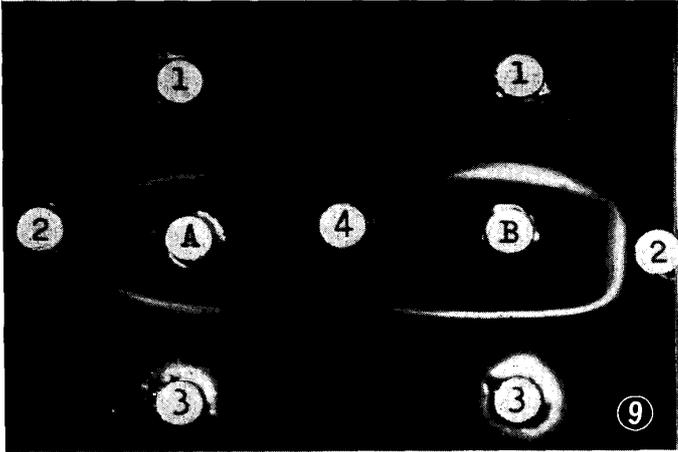


PLATE II

Figures 17~19, center well A; serum from CF#1 mouse, 10 mo. post infection and center well B; antiEmc

Fig. 17 Peripheral well 1; crude Emc (70°C, 10 min), well 2; crude Emc (70°C, 20 min), well 3; crude Emc (raw) and well 4; crude Emc (56°C, 30 min)

Fig. 18 Peripheral well 1; crude Emc (100°C, 15 min), well 2; crude Emc (raw), well 3; crude Emc (raw 1 : 2) and well 4; crude Emc (100°C, 5 min)

Fig. 19 Absorption test
Peripheral well 1; crude Emc (70°C, 10 min), well 2; crude Emc (70°C, 20 min), well 3; crude Emc (raw) and well 4; crude Emc (56°C, 30 min)

Fig. 20 Absorption test
Center well A; antiEmc and center well B; serum from CF#1 mouse, 10 mo. post infection
Peripheral well 1; crude Emc (100°C, 15 min), well 2; crude Emc (raw), well 3; crude Emc (raw 1 : 2) and well 4; crude Emc (100°C, 5 min)

Figures 21~23, peripheral well 1; antiEmc, well 2; antiNML, well 3; antiNMS and well 4; serum from CF#1 mouse, 10 mo. post infection

Fig. 21 Center well A; crude Emc (56°C, 30 min) and center well B; crude Emc (raw)

Fig. 22 Center well A; crude Emc (70°C, 20 min) and center well B; crude Emc (70°C, 10 min)

Fig. 23 Absorption test
Center well A; crude Emc (raw) and center well B; crude Emc (100°C, 15 min)

Fig. 24 Center well A; antiEmc (absorbed by NMS & NML)
Peripheral well 1; NML, well 2; NMS, well 3; fractionated Emc (raw) and well 4; fractionated Emc (100°C, 15 min)

