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Purification of *Toxoplasma* Trophozoites from the Peritoneal Exudate of Infected Mice

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Introduction

Since the discovery of *Toxoplasma gondii* by Splendore in Brasil in 1908, and Nicolle and Manceaux in North Africa in the same year, studies on this protozoa have been carried out by numerous investigators in many respects. Most of them, however, deal with its morphology, immunology, epidemiology, and clinical aspects. Biochemical researches have appeared very few. Therefore, until now, the physiological characteristics of this protozoa remain mostly unknown. This is mainly due to the fact that purification of *Toxoplasma* from the peritoneal exudate of infected animals for the purpose of biochemical investigations has been very difficult being disturbed by the host cellular components such as peritoneal macrophages. Purification of the organism has been reported by several investigators such as Westphal (1958), Niki (1959), Tsunematsu (1960), Fulton and Spooner (1960), Lyche and Lund (1964), Neimark and Blaker (1967), and Remington *et al.* (1970). Although all of these methods were carefully re-examined in our laboratory, none of them presented any satisfactory results.

The present report deals with a new purification method of *Toxoplasma* from the peritoneal exudate of mice using centrifugation with tris (tris hydroxymethyl aminomethane)-sucrose-EDTA (ethylene diamine tetra-acetate) solution, and the combination of the above-mentioned solution and anti-mouse peritoneal cell serum (AMPCS).

Materials and Methods

RH strain of *Toxoplasma* retained in our laboratory was used throughout the experiment. All of the reagents used were special grade. Disodium salt was employed as the source of EDTA ion. Compositions of tris-sucrose-EDTA solution of different specific gravities are indicated in Table 1.

Table 1 Compositions of tris-sucrose-EDTA solution

(1) Specific gravity 1.072	
Sucrose	35.8738 g
Disodium EDTA	0.1 g
Tris (hydroxymethyl aminomethane)	0.0262 g
Distilled water to make 500 ml	
(2) Specific gravity 1.074	
Sucrose	36.8738 g
Disodium EDTA	0.1 g
Tris	0.0262 g
Distilled water to make 500 ml	

Collection of the organism

Trophozoites of the protozoa, about one million in number, were inoculated into the peritoneal cavities of 10-15 mice. Four days later, these mice were killed in chloroform vapour, and small incisions were made on the abdominal walls to expose the peritoneum. Then, in each mouse, 5 ml of sterilized 0.85 % NaCl solution was injected into the peritoneal cavity not so as to injure the blood vessels, and the same procedures were repeated once more. Ultimately, about 10 ml of the peritoneal fluid containing the protozoa and host peritoneal cells was obtained in each mouse, and the fluid from all the mice was pooled at 4°C.

Centrifugation

Centrifugation in the purification process

was made using high speed cold centrifuge of Kubota Seisakusho Co. Ltd. (Model KR-6 P). The maximum volume of the centrifugal tube employed in the experiment was about 10 ml, and the height of the tube was about 7.5 cm. The radius of the rotar was 9.7 cm.

Method of making anti-mouse peritoneal cell serum (AMPCS)

(1) Mouse peritoneal cell antigen

Sterilized glycogen suspended in normal saline at 0.06 % was injected 2.0 ml each into the peritoneal cavities of 20 mice. Three days after the injection, the peritoneal exudate containing the peritoneal cells were collected by the same method as described above except for use of 5 ml of Hanks balanced salt solution instead of normal saline.

The solution collected was then centrifuged at 3,000 rpm for 5 minutes. The peritoneal cells collected in the sediment were suspended again in cold Hanks balanced salt solution, and washed three times. The final suspension containing a determined number of the cells was injected into the rabbits as mouse peritoneal cell antigen fluid, with or without Freund's incomplete adjuvant by case.

(2) Mouse spleen sell antigen

The spleens removed from 3 mice were pooled in sterilized cold 0.85 % NaCl solution, crushed gently, and agitated with a capillary pipette in order to obtain homogeneous suspensien of spleen cells. The suspension was then filtrated through stainless mesh, and washed three times with sterilized Hanks balanced salt solution. The final sediment was re-suspended in 5.0 ml of the solution, and after counting the number, it was mixed with the peritoneal cells collected in the way mentioned above. The mixed suspension was injected to the immunized rabbits intravenously as the booster injection.

(3) Process of immunization of rabbits

Immunization was made for two rabbits, weighing about 2 kg each, which showed negative reaction by *Toxoplasma* H-A test immediately before use. The rabbits were immunized four times by intramuscular injection of the mouse peritoneal cell antigen fluid with adjuvant. Later, the mixed antigen fluid without adjuvant was injected intravenously as the booster. Detailed explantions of the immunization process were indicated in Table 2. Antibody titre of the rabbits was examined by agglutination test for mouse peritoneal cells one week after the final immunization. In case that the titre elevated more than 4,096×, whole blood was extracted from the bilateral carotid artery three days after the titration. Then the serum was separated, inactivated, and stored until use.

Evaluation of purity of the final suspension

Purity of the final products of the experiment was examined both in fresh and stained preparations. In fresh preparations, numbers of the host cells and organism were counted respectively using hemocytometer,

Table 2 The process of immunization for making anti-mouse peritoneal cell serum

No. of days after the start of immunization	No. of peritoneal cells injected ($\times 10^7$)	Adjuvant	No. of spleen cells injected ($\times 10^8$)	Adjuvant
1	1.51	+		
2	3.03	+		
16	6.06	+		
17	7.58	+		
24	0.60	−	3.11	−
25	3.56	−	4.88	−
26	2.31	−	2.86	−
33	0.68	−	3.91	−
34	3.38	−	2.04	−
35	4.41	−	3.84	−

and then the ratio of them was calculated. In addition, microphotographs of the fresh and stained preparations by alkaline methylene blue were taken.

Evaluation of pathogenicity of purified Toxoplasma

Pathogenicity of the purified *Toxoplasma* to mice was compared with that of the organism collected directly from the peritoneal exudate of the infected mice. The determined number of the purified organism was inoculated into the peritoneal cavities of 5 clean ICR mice. The almost same number of washed trophozoites of RH strain which were collected from the peritoneal exudate of infected mice was inoculated into the peritoneal cavities of control mice. Then the survival period of the experimental mice was compared with that of the control mice in order to evaluate suspectable changes in biological activities of the purified organism.

Results

After many trials, principal method for purification was established. Steps of the purification procedures thus determined are as follows:

(1) Collect the organism as well as the peritoneal cells of mice by centrifugation
(2) Wash the sediment with buffered isotonic solution
(3) Suspend the sediment in tris-sucrose-EDTA solution or mixture of tris-sucrose-EDTA solution and AMPCS using the centrifugal tube
(4) Keep the tube standing still for 30–180 minutes
(5) Centrifuge the tube
(6) Collect the upper part of the supernatant using a capillary pipette.
(7) Centrifuge the supernatant collected
(8) Wash the sediment with buffered isotonic solution repeatedly

The final sediment contains the organism freed from other cells almost completely. According to the principal method mentioned above, some improvements were made in each step as stated in the following explanations.

Purification with the use of tris-sucrose-EDTA solution only

Tris-sucrose-EDTA solution of specific gravity (S.G) 1.072 was employed in step (3) of the principal method. The sediment, *Toxoplasma* and mouse peritoneal cells, obtained in step (2) was suspended in 5.5 ml of tris-sucrose-EDTA solution of S.G 1.072, and the tube containing the suspension was kept standing for 180 minutes at 4°C. After the cold centrifugation in step (5) at 1,200 rpm for 7 minutes, upper three-fourth of the supernatant was carefully transferred into the centrifugal tube using a capillary pipette, and then centrifuged at 3,000 rpm for 10 minutes. The sediment thus obtained was washed three times by cold centrifuge at 3,500 rpm for 5 minutes with 1.2 ml of tris-HCl buffer (pH 7.2) containing sucrose in 0.25 M. By microscopical examinations of the final sediment thus obtained, it was revealed that *Toxoplasma* trophozoites were obtained almost completely free from the host cells as shown in Fig. 1–2.

Purification with the use of combination of tris-sucrose-EDTA solution and AMPCS

Tris-sucrose-EDTA solution of S.G 1.074 was used instead of the solution of S.G 1.072 in step (3) of the principal method mentioned above. *Toxoplasma* and mouse peritoneal cells collected by centrifugation in step (2) were suspended in 7.5 ml of the solution of S.G 1.074. The suspension was added with 0.5 ml of AMPCS and mixed thoroughly. The centrifugal tube containing the suspension was kept standing for 30 minutes at 37°C without moving. After the cold centrifugation in step (5) at 1,000 rpm for 3 minutes, upper four-fifths of the supernatant was carefully taken by a capillary pipette, centrifuged, and washed in the same way as in the former method.

During these procedures described above, centrifugation in step (5), and step (8) is important. In step (5), only *Toxplasma* is separated from the suspension containing both the organisms and the host cells. In step (8), cell debris produced by rupture of

the host cells is eliminated from the suspension. Purified *Toxoplasma* by this method are demonstrated in Fig. 3-4.

Recovery rate of the organism and elimination rate of the host cells

Recovery rate of the organism in the final product from the mouse peritoneal exudate initially used was calculated in each method. In the method in which tris-sucrose-EDTA solution only was used, the recovery rate of the organism was about 25 %. On the other hand, the method of combination of tris-sucrose-EDTA solution with AMPCS recovered the organism in almost 40 %.

In addition, the elimination rate of the host cells in the final product was also calculated. In both methods, the elimination rate of the host cells reached more than 99.9 %.

Ratio of Toxoplasma *to the host cells in the final suspension*

Number of the host cells which contaminated slightly the final suspension was compared with that of *Toxoplasma* in the several cases of both methods. The result is shown in Table 3. The ratios of the host cells to *Toxoplasma* were 1 : 3,720 in maximum, and 1 : 21,800 in minimum, being 1 : 10,243 in average. Microphotographs (Fig. 1-4) also demonstrate no presence of the host cells among numerous *Toxoplasma* trophozoites.

Pathogenicity of purified Toxoplasma

Pathogenicity of the purified *Toxoplasma* to mice was compared with that of the organism used for the routine serial transfer. As is shown in Table 4, no difference in survival period was recognized between experimental and cotrol mice. This fact suggests that no change occurred in biological activity of the organism during the purification procedures.

Discussion

As described in the introduction, Westphal (1958), Niki (1959), Tsunematsu (1960), Fulton and Spooner (1960), Lyche and Lund (1964), Neimark and Blaker (1967), and Remington *et al.* (1970) reported successful results in the purification of *Toxoplasma* trophozoites. Westphal (1958) obtained pure *Toxoplasma* suspension by the method in which ultrasonication destroyed only the host cells suspended in citrate buffer saline keeping the organisms intact. Niki (1959), and Tsunematsu (1960) sonicated or ultrasonicated the initial mixture

Table 3 Number of *Toxoplasma* and the host cells in the final suspension of various samples

No. of experiment	No. of *Toxoplasma* ($\times 10^4$)	No. of host cells ($\times 10^4$)	Ratio
1	3,720	1	3,720
2	10,420	2	5,210
3	2,150	0	
4	7,690	1	7,690
5	24,190	4	6,047.5
6	35,280	4	8,820
7	24,300	2	12,150
8	25,120	2	12,560
9	21,800	1	21,800
10	20,000	1	20,000
11	3,520	0	
12	6,170	0	

* 1- 5, In the case of using tris-sucrose-EDTA solution only.
6-12, In the case of using the combination of tris-sucrose-EDTA solution and AMPCS.

Table 4 Pathogenicity of purified *Toxoplasma* to mice

(1) In the case of using tris-sucrose-EDTA solution only

	No. of mice examined	No. of mice survived after the inoculation					
		1 (day)	2	3	4	5	6
Control	5	5	5	5	4	0	0
Experiment	5	5	5	5	5	1	0

(2) In the case of using the combination of tris-sucrose-EDTA solution and AMPCS

	No. of mice examined	No. of mice survived after the inoculation						
		1 (day)	2	3	4	5	6	7
Control	5	5	5	5	5	5	2	0
Experiment	5	5	5	5	5	5	2	0

of *Toxoplasma* and the host cells, and thereafter the suspension was digested by trypsin solution to destroy the partially injured host cells. These two procedures resemble each other to some extent. Niki (1959) and Tsunematsu (1960) stated in their papers, that the recovery rate of the organism was 30–60 %, and the elimination rate of the host peritoneal cells was about 100 %. But the method pursued in our laboratory resulted in failure because some host sells appeared to be intact even after the process was over. Furthermore morphological changes were observed in some of the organism. It is probable that the techniques such as ultrasonication or trypsin digestion cause some changes in morphology and physiology of the organism. This is noticed in Tsunematsu's report (1960) that the purified *Toxoplasma* decreased the infectivity to mice very much. With regard to the damage of trypsin digestion to the organism, Motomura (1968) showed complete death of *Toxoplasma* trophozoites (RH strain) by the digestion of 0.5 % trpysin solution. Moreover Motoyoshi *et al.* (1968) reported the loss of infectivty of low virulent *Toxoplasma* isolated from a pig, by the digestion of 0.5–5.0 % trypsin solution for 30–60 minutes. These experimental results indicate that the digestion by trypsin solution must injure the organism.

Fulton and Spooner (1960) reported a purification method using filtration with a sintered glass filter of pore size 15–35 μ and anti red blood cell serum to remove contamination with the cells. Thereafter Neimark and Blaker (1967), and Remington *et al.* (1970) also used a method almost same as Fulton's one, and the former authors observed slight contamination of red blood cells. In our laboratory, purification of *Toxoplasma* trophozoites was tried several times according to this filtration method, but any satisfactory results could not be acquired. On the basis of the results obtained by the filtration experiments in our laboratory, the present author tried to purify *Toxoplasma* trophozoites by filtration with a menbrane filter of pore size 8 μ (GENERAL ELECTRIC, U.S.A.). *Toxoplasma* (RH strain) and mouse peritoneal cells collected by centrifugation were suspended in suitable buffer saline (pH 7.2), and filtrated through the menbrane filter. The filtrate, however, contained many host peritoneal cells as well as red blood cells involved in the initial suspension. It seems to be curious in a sense that in the case of a filter of pore size 15–35 μ, host cells can be removed, whereas in the case of pore size 8 μ, these cells still remained in the filtrated sample. It is very difficult to explain the fact like this. The difference of thickness of the filters used may be one of the causes of this disagreement. In addition, intensity of suction power in the filtration may relate to this problem.

Lyche and Lund (1964) purified the organism by centrifugation only. In the report they described that more than 90 % of the host cells contained in the initial suspension could be removed. However, 10 % of the host cells still remaining in the purified semple must be a big barrier in performance of biochemical experiments.

The method described in the present report is based on the use of the difference of the specific gravity between *Toxoplasma* and mouse peritoneal cell. In addition, AMPCS is used for agglutinating host peritoneal cells. This antiserum seems to contain several sorts of antibody, i.e. anti-macrophage antibody, anti-lymphocyte antibody, anti-white blood cell antibody, and anti-red blood cell antibody. Therefore, any kind of cells in the peritoneal exudate of mouse infected with RH strain *Toxoplasma* can be agglutinated.

It is widely accepted that the specific gravity of red blood cell is 1.090, and that of white blood cell is 1.065–1.070. The specific gravity of macrophage which is most prominent among the cells in the peritoneal exudate of mouse infected with the organism seems to be almost the same as that of white blood cell, because these two kinds of cells do not contain hemoglobin. Therefore, an isotonic solution of specific gravity about 1.070 is needed to separate these host cells. On the basis of these demands, sucrose was used. EDTA was employed for protecting the natural agglutination and clumping of the organism. Tris (tris hydroxymethyl aminomethane) was used for adjusting pH of the solution and gaining buffer action. The reason why mouse spleen cells were also utilized as the antigen to make AMPCS is in existence of common antigens between mouse peritoneal cells and spleen cells.

As explained in the result, about 25–40 % of the organism in the initial suspension, occationally more than 50 %, is recovered as the final product, and the contamination with host peritoneal cells and their debris is removed almost completely.

In additon, the biological activity of the purified organism seems to be satisfactorily preserved as it is represented by the fatality of ICR mice inoculated. Therefore, the purified organism by means of this procedure appears to be sufficienty available for the biochemical investigations also. Some problems may exist in the method described here, i.e. probably only the extracellular trophozoite in the initial suspension is recovered, the so-called *Toxoplasma* hostile factor may be present in AMPCS and tris-sucrose-EDTA solution in this experiment is slightly hypotonic.

Toxoplasma is an obligate intracellular parasite which never be able to survive or proliferate without living cells. The metabolic activity of the organism must be dependent partially on the host cells. Therefore, when the organism is present in extracellular space, its metabolic pathway may not be the same as in intracellular space. Judging from this possibility, rash conclusions in the biochemical researches about the metabolism of *Toxoplasma* must be refrained.

Concerning the so-called *Toxoplasma* hostile factor, many authors have reported the presence of the factor of properdin like nature in the fresh serum of man, calf, and rabbit etc. Kobayashi *et al.* (1967), however, stated that the activity of the factor was repressed by addition of 0.4 % EDTA or 6 % Alsever solution, or by heating the serum at 56°C for 30 minutes. Therefore, it is unlikely that AMPCS used in the present experiment contains the so-called *Toxoplasma* hostile factor. On the other hand, however, Strannegärd (1967) reported that only 20 % of the initial activity of the factor in the fresh serum of rabbit could be repressed by heating at 60°C for 30 minutes.

In the present experiment, purified orgnism was stained by alkaline methylene blue which is routinely employed in Sabin-Feldman's dye test. When anti-*Toxoplasma* antibody is present sufficiently in the reaction mixture of this test, the organism can not be stained by the dye. However, as known in the microphotographs illustrating the purified organism stained by the dye, *Toxo-*

plasma was well stained. Therefore, in consideration of preservation of its pathogenicity to mouse, it is reasonable to suppose that the effect of AMPCS on the organism is nothing or negligible. Furthermore, these experimental facts appear to be peritnent for being confident of the absence of impairment by using slightly hypotonic trissucrose-EDTA solution, and by the components of the solution.

Summary

Trophozoites of *Toxoplasma* (RH strain) were purified from the peritoneal exudate of mice infected experimentally. The purified organism free from mice peritoneal cells almost completely was obtained using centrifugation with tris-sucrose-EDTA solution of specific gravity 1.072, and the combined use of the above-mentioned solution of specific gravity 1.074 and anti-mouse peritoneal cell serum. In the method in which tris-sucrose-EDTA solution only was used, the recovery rate of the organism was about 25 %. On the other hand, the method of the combined use of tris-sucrose-EDTA solution and anti-mouse peritoneal cell serum recovered the organism in almost 40 %, occationally more than 50 %. In both cases the elimination rate of the host cells reached more than 99.9 %. Pathogenicity of purified organism thus obtained to mice was well preserved.

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感染マウス腹水からのトキソプラスマ栄養型虫体の純粋分離

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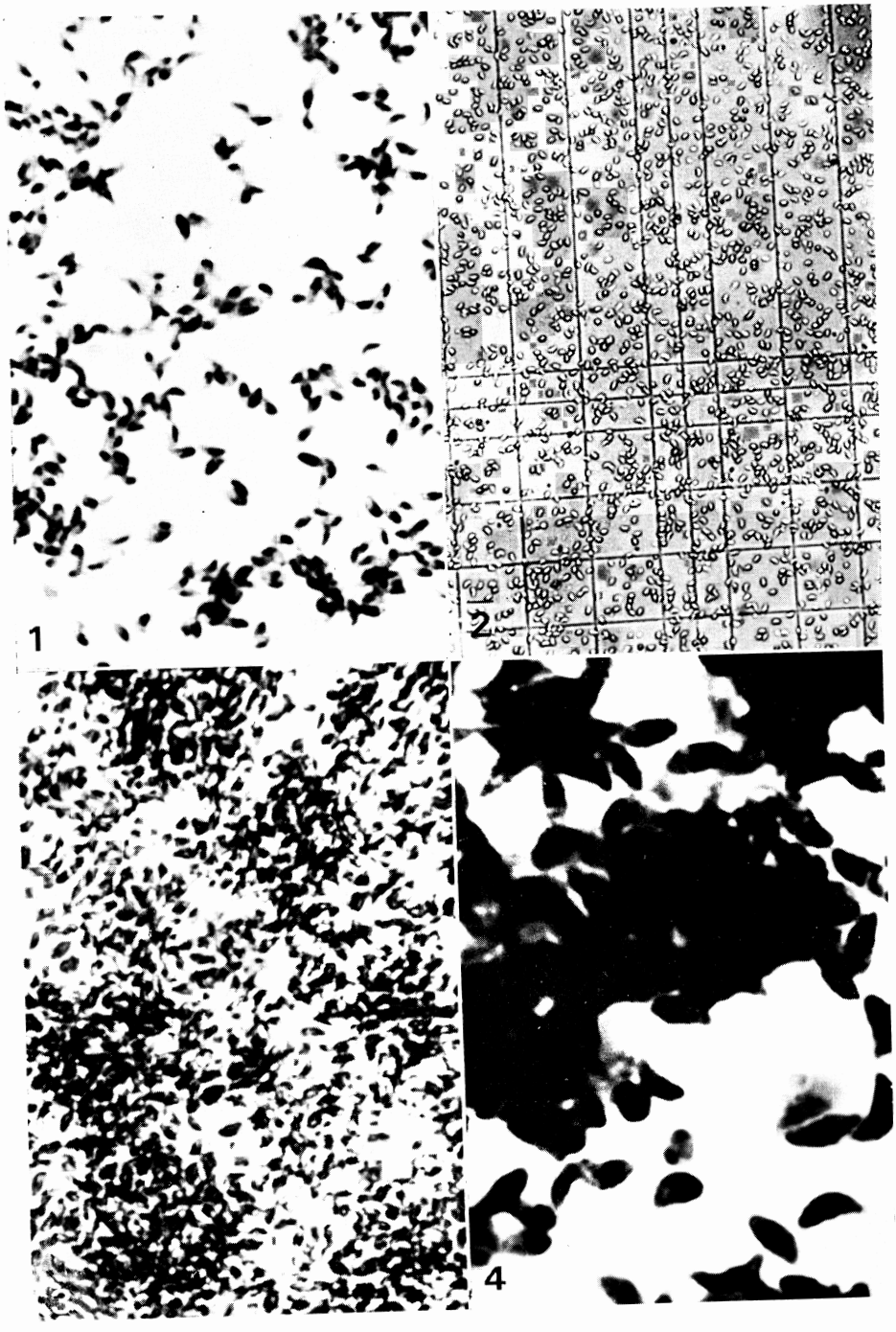
トキソプラスマの生理学的研究を目標としてその栄養型虫体の純粋分離を行つた．使用株は当教室で保存しているRH株である．RH株を約100万個，10～15匹の市販マウスの腹腔内に注射し，4日後クロロフォルムにてマウスを殺し滅菌生理食塩水にて腹腔内を洗い，原虫とマウス腹腔内細胞の浮遊液を得た．この浮遊液を遠沈して沈渣に原虫とマウス腹腔細胞を回収し，5.5～6.0 mlの比重1.072の Tris-Tucrose-EDTA Solution に浮遊し約3時間，4°C に放置した．その後1,200 rpm, 7分間遠沈し，上清の¾を回収しこれを遠沈，沈渣を3回 Tris HCl buffer, Sucrose 0.25 M, pH 7.2にて洗滌した．こうして得られた最終浮遊液はほとんどマウス腹腔内細胞を含まず，純粋なトキソプラスマ浮遊液と見なすことができた．更に他の方法として，同様に集めた原虫とマウス腹腔内細胞を比重1.074の Tris-Sucrose-EDTA Solution, 7.5 ml に浮遊し，これに予め作成しておいたウサギ抗マウス腹腔内細胞血清（抗体価1：4096×）を0.5 ml 加え37°C に30分間放置して，原虫をより多く回収することを企図した．この場合，37°C に30分間放置後，1,000 rpm, 3分間遠沈し，上清の⅘を回収し，前の方法の場合と同様に遠沈，洗滌した．このようにして得られた浮遊液も殆んどマウス腹腔細胞を含まなかつた．原虫の回収率は前の方法の場合は24％前後，後の方法の場合は40％前後であつた．又いずれの方法でも99.9％以上のマウス腹腔細胞を除去することができた．分離された原虫の生物活性はマウスに対する病原性を示標として検討されたがいずれの場合も低下は認められなかつた．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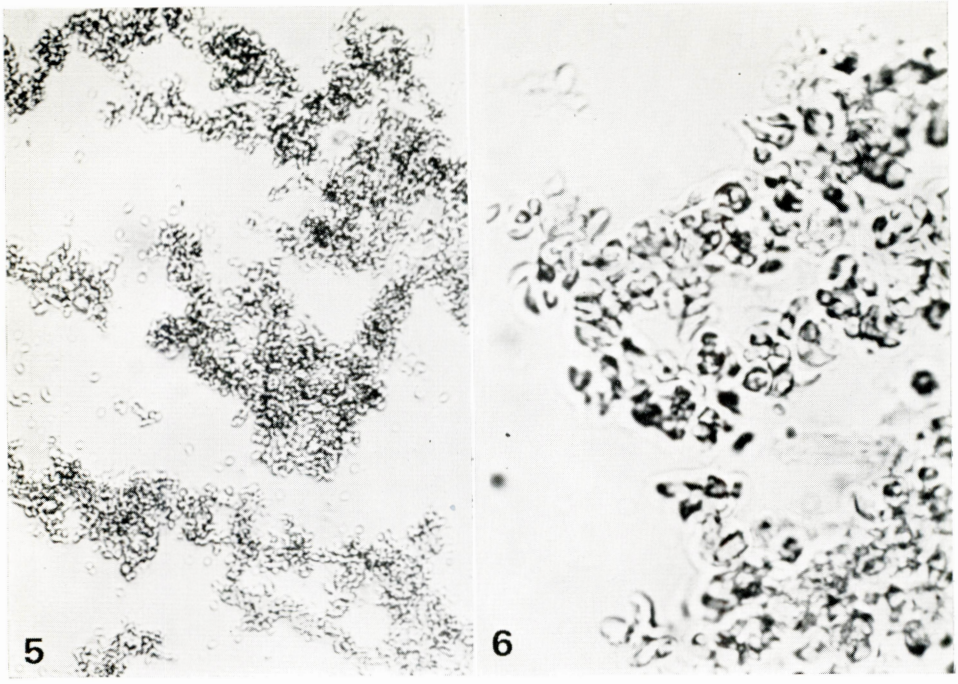

Explanation of Figures

Fig. 1 Stained preparation of purified *Toxoplasma* by means of tris-sucrose-EDTA solution only. (× 400)

Fig. 2 Fresh preparation of purified *Toxoplasma* by means of tris-sucrose-EDTA solution only. (× 100)

Fig. 3 Stained preparation of purified *Toxoplasma* by means of the combined use of tris-sucrose-EDTA solution and AMPCS. (× 400)

Fig. 4 High magnification of Fig. 3. (× 1,000) No host peritoneal cells are observed in Fig. 1–4.

Fig. 5 Mice peritoneal cells agglutinated by AMPCS. (× 100)

Fig. 6 High magnification of Fig. 5. (× 400)