

COMPARATIVE EVALUATION OF TISSUE FRACTIONS AND
METABOLIC PRODUCTS OF TAENIID TAPEWORMS AS ANTIGENS
IN THE INDIRECT HEMAGGLUTINATION TEST FOR HYDATID
DISEASE

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ECHINOCOCCUS GRANULOSUS

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ABSTRACT

The object of this investigation was to study under as nearly standard conditions as possible, various biological, physical and chemical properties of the antigenic material which is active in the indirect hemagglutination test for hydatid disease.

It was found that the host source of antigen is of no significance in the indirect hemagglutination test as long as the cyst fluid is obtained from highly fertile, healthy cysts.

Large infertile cysts in cattle were, in all probability, originally fertile but the scolices had subsequently degenerated. Fluids from such cysts were non-antigenic or gave very low serum titers. Infertile cyst fluid from secondary infections of mice where the scolices had not yet formed were sometimes antigenic, suggesting that maybe certain cells of the germinal membrane had become differentiated to antigen producing cells. Perhaps differentiation had not yet taken place in the case of some such pools which were not antigenic.

Heating the hydatid cyst fluid for 25 minutes at 56°C and 70°C did not inactivate the cyst fluid in the indirect hemagglutination test. Heating it for 25 minutes at 80°C gave doubtful results, while heating for 5 minutes at 80°C reduced its antigenic activity. Heating hydatid cyst fluid at 100°C for 5 minutes inactivated it completely.

Filtering the hydatid cyst fluid through Seitz filters or exposing it to Seitz filter pads inactivated the fluid in the indirect hemagglutination test indicating adsorption rather than filtration loss.

Saline extracts of the scolices did not possess any antigenicity when tested by the indirect hemagglutination test and did not adsorb antibody from the standard serum. In addition, lysing the scolices within highly antigenic hydatid cyst fluid in an effort to enhance its antigenic titer surprisingly destroyed the antigenic activity already possessed by the fluid. This suggested the possibility that the scolices had released non-specific substances which, however, were competitively adsorbed with the antigen on the erythrocytes during sensitization. Evidence in support of this hypothesis was obtained by use of non-antigenic protein solutions as antigen diluents.

Results of the indirect hemagglutination test on lyophilized fractions of Echinococcus granulosus were that the dialysis precipitate, and trichloroacetic acid precipitate proved to be active but not as active as the original hydatid cyst fluid. The alcohol precipitate was inactive indicating that the antigen is not a mucopolysaccharide. This is supported by the fact that Taenia hydatigena cyst fluid which is free from hydatid mucopolysaccharide was equally antigenic in the indirect hemagglutination test.

In fact, one of the most interesting findings in this study was that the indirect hemagglutinating antigen is group specific in that Taenia hydatigena fluids substituted for hydatid cyst fluids in the test.

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INTRODUCTION

Since its introduction by Garabedian et al (1957a), the indirect hemagglutination test for hydatid disease has seen ever increasing use. There have been few studies, however, on the physical properties and chemical characteristics of the antigen for this test. Several investigations (Garabedian et al, 1957ab, 1959; Knierim and Wiedmann, 1962; Kagan et al 1959) have shown the indirect hemagglutination test to be more specific and sensitive than the complement fixation and precipitin tests, and in some instances, than the Casoni intradermal test. In addition, it is cheap and relatively simple to perform. Were the specific antigen isolated and identified and its chemical and physical properties known, it is likely that the indirect hemagglutination test would be even more useful in the diagnosis of hydatid disease than at present. Most sero-diagnostic work on helminthiases is plagued by the crude nature of antigens employed (Kent 1963).

In this study the antigenic activities of hydatid cyst fluid from various mammalian sources were compared and attempts were made to fractionate them into active and inactive portions. Additional studies were done to define, in part, the chemical and physical properties of the indirect hemagglutination test antigen.

A. Hydatid Disease

Hydatid disease is a serious infection of man caused by the larval stage of Echinococcus granulosus. It has been known and described since ancient times (Hoeppli, 1959). This disease manifests itself in the form of a continually growing hydatid cyst in various organs. The distribution of hydatid disease is nearly world-wide but is of greatest importance in areas where sheep-raising is extensive. In regions such as New Zealand, Argentina and parts of East Africa infection of the intermediate hosts reaches more than 50 per cent; however, human infection is always much less (Gemell 1960).

The adult worms of Echinococcus granulosus vary in length from 1.5 to 6 mm and are usually composed of five parts: the scolex which has four suckers and a rostellum with two rows of hooks, a short neck, a single immature proglottid, a single mature proglottid which contains the reproductive organs and a terminal gravid proglottid consisting almost entirely of a uterus distended with eggs.

The adult worms are found attached to the wall of the small intestine of dogs and several wild carnivores. The eggs are passed in the feces of these animals either with the gravid proglottid or free following rupture of the gravid proglottid in the intestines. If the eggs are ingested by a suitable intermediate host (cattle, sheep, pigs, horses, camels, moose, deer or man), they hatch in the duodenum liberating motile oncospheres (Meymarian 1961) which penetrate the intestinal mucosa and enter the blood stream. They are usually filtered out and develop in the liver (approximately 50-69 per cent)

or lungs (approximately 7.4 - 11.9%) but may occasionally appear in any tissue.

The oncosphere imbibes water and forms a small cyst consisting of germinal membrane composed of one layer of cells, and a thick, elastic outer laminated membrane. The host attempts to isolate the cyst by laying down in most organs a thick fibrous membrane gradually merging into normal tissue. The entire cyst is filled with fluid and grows continuously by imbibition of water. When the cyst is approximately one cm in diameter, brood capsules form on the germinal membrane, within each of which up to 20 to 40 scolices form.

These scolices are ingested by suitable carnivores when they feed on the viscera of infected animals. The cycle is completed when the scolices evaginate and grow to maturity within the small intestine of the carnivore.

Symptoms are usually lacking in domestic animals due to their short life span as compared to the usually long period required for development of a hydatid cyst to large size. In human beings cysts may go undetected for as long as 5 to 20 years, or until they reach a size which interfered with the function of a vital organ.

Diagnostic methods differ depending upon the organ infected and the location and size of the cyst. X-rays contribute most to diagnosis of hydatid cysts in lungs and bones. Serological tests which have been used in diagnosis include a precipitin test and a complement-fixation test, and more recently an indirect hemagglutination test, a bentonite flocculation test and a polystyrene latex test. An intradermal test is also used.

The only presently available treatment is the complete excision of the cyst. This is not possible or successful, of course, in all locations.

Infection can be controlled by preventing the carnivorous hosts from eating infected viscera. In endemic areas, dogs which are the principle source of infection to man, should be periodically examined and treated for tapeworms.

B. Chemical Composition of Hydatid Cyst Fluid

The chemical composition of hydatid cyst fluid and tissues of the parasite have been the subject of several investigations. As early as 1923, Mazzacco analyzed bovine hydatid cyst fluid by clinical chemical tests for serum, and found glucose, protein, cholesterol, amino acids, fatty acids and various other chemical components.

Other investigators, Lemaire and Ribère (1935), found the concentration of cholesterol was not constant in hydatid fluid; it appeared to be greater in hepatic hydatid cyst fluids than in cysts from other sites. They also suggested that cyst fluid albumin was probably lost during their assay procedure by proteolytic fermentation.

In later investigations on the albumin content of hydatid cyst fluid, Couadounis and Polydorides (1936) found a low content of albumin which was independent of the organ containing the cyst. In contrast they found the sugar content varied greatly depending on the organ in which the cyst was located. They postulated that sugar could pass from the liver through the cyst membrane and into the hydatid cyst fluid.

The work of Schwabe (1959) and Schwabe, et al (1961) on the permeability of the hydatid cyst wall has shown it to be freely permeable to small molecules such as sugars. In addition to protein of uncertain origin and certain ions and small molecules which may be contributed to the cyst fluid by the host rather than by the parasite, few metabolic products of cyst origin have been identified. The only

such product which has been at all characterized chemically is a galactose- and glucosamine-containing mucopolysaccharide (Kilejian et al, 1961, 1962; Schwabe and Koussa, unpublished studies).

Magath (1959) and Goodchild and Kagan (1961) found that concentrated hydatid cyst fluid from various sources gave an electrophoretic pattern for protein somewhat similar to that of serum. Four distinct protein components have been obtained by Schwabe and Koussa (unpublished work), two of which correspond in electrophoretic mobility to serum albumin and gamma globulin. According to Kagan (personal communications) some of this protein is of host origin. Biocca and Lorensetti (1960) reported higher protein content in fertile than in infertile cysts.

C. Chemical Composition of Hydatid Cyst Tissues

Hydatid tissues have been more thoroughly studied chemically. Cameron (1926) demonstrated a diastase in the cyst walls and traces of proteolytic enzymes and lipase. He suggested that the presence of glycogen is an important factor in the metabolism of the parasite. Čmelik (1952a) showed that the unsaponifiable fraction of cystic membranes of Echinococcus granulosus from pig livers was cholesterol. Half of the fatty acids were not soluble in water and assumed to be oleic acid. (Kilejian et al (1962) suggested the presence of both cholesterol and lecithin in extracts of the cyst wall. Frayha and Durr (unpublished work), have since shown that the cholesterol of the parasite is of host origin and is not synthesized by the parasite from acetate.

In further investigations on the membranes, Čmelik and Briski (1953) found a nucleoprotein whose nucleic acid contained neither ribose nor desoxyribose but instead a hexose. Different nucleoproteins were obtained from the scolices and also a glycoprotein. The same or another protein-containing polysaccharide extracted from scolices contained as its sugar components glucose, galactose and glucosamine.

Histochemical observations on the hydatid cyst wall demonstrated that the laminated membrane contains a periodic acid Schiff-positive substance other than glycogen, and that it is also present in the germinal membrane and scolices (Kilejian et al, 1961). These workers, in contrast to Čmelik, found histochemical evidence of DNA in the germinal membrane nuclei.

Agosin et al (1957) obtained two types of "polysaccharides" from Echinococcus granulosus scolices. One was glycogen which accounted for nearly 20 per cent of the dry weight of the scolices. The other was composed of galactose and glucosamine and possibly glucose. Kilejian et al (1962), using infrared spectrophotometry, disclosed glycogen in the membrane of Multiceps multiceps and Taenia hydatigena, while the laminated membrane of Echinococcus granulosus indicated a different polysaccharide. This was shown by electrophoretic and chromatographic studies to be a mucopolysaccharide, containing galactose and glucosamine as its only sugar components. It was found also in the scolices and cyst fluid and was no doubt identical to the second "polysaccharide" from scolices of Agosin et al (1957). Lorvik and Moriconi (1956) had also reported a neutral mucopolysaccharide in the cyst membranes of echinococcus cysts.

D. Nature of Antigen-Antibody Relationships

Antibodies are considered to be proteins or inseparably associated with proteins. They are located in the globulin fraction of the blood. The formation of antibodies occurs in various organs, particularly the spleen, liver, bone marrow and lymph nodes. An antibody is capable of combining only with the antigen which stimulated its production or some very closely related type of antigen. This specificity is reminiscent of the specificity shown by enzymes for their substrate.

Antigens are usually proteins but some polysaccharides can act as complete antigenic agents. A few proteins of relatively low molecular weight possess little or no antigenicity. Starches, dextrans and glycogens have been reported to be non-antigenic under normal conditions, but sometimes a polysaccharide attached to a protein may influence the serological specificity of the protein. Lipids and nucleic acids are antigenic only when combined with protein. Denaturation diminishes or completely destroys the antigenic activity of most proteins.

Some animal and bacterial lipids and polysaccharides are found to be serologically active in the test tube, but do not induce the formation of antibodies when injected into animals. Such substances are known as haptenes. These are further divided into complex and simple haptenes. Complex haptenes are those which combine specifically with antibody preparations to yield a precipitate or other visual reaction. Simple haptenes do not react visibly with antibody preparations, but somehow combine with the antibody and prevent its consequent precipitation by the complete antigen.

The reaction between antigens and antibodies, can be detected by agglutination, precipitation, lysis, complement fixation and other reactions or by an anaphylactic reaction in the living animal (Carpenter 1956, Hardin 1961).

E. Sero-Diagnosis of Hydatid Disease

Several tests have been proposed for the diagnosis of hydatid disease. One of the simplest of these is the intradermal test. It was introduced by Casoni in 1911. It consists of injecting the infected individual intradermally with 0.5 cc of bacteriologically sterile fluid from fertile hydatid cysts to which has been added one drop of phenol per 20 cc of the fluid. A control is used consisting of an isotonic NaCl solution with the same amount of phenol added. The reaction is read three to twelve hours later and the presence of a red wheal at the site of the injection indicates a positive reaction while the control gives no reaction. Magath (1959) modified Casoni's test slightly by preserving the fluid with merthiolate and as a control used merthiolated saline solution. The typical response was observed in five to fifteen minutes using lesser quantities of hydatid cyst fluid.

The precipitin test was introduced by Fleig and Lisbonne (1907). In the precipitin test the antigens are prepared in the same manner as for Casoni's test except phenol is not added. The serum from the patient is used without dilution or heating and must be crystal clear. The antigen is serially diluted. The serum is carefully introduced onto capillary tubes and the antigen solution is then carefully layered onto the surface of the serum. If the serum is very active a precipitate appears at the interface almost immediately. Precipitation is usually complete by the end of two hours at room temperature, or overnight in the refrigerator.

The bentonite flocculation test was first described by Bozicevich et al (1951) for use in diagnosing trichinosis. This technique was adapted

for the diagnosis of hydatid disease in man and animals by Norman et al (1959). Fischman (1960) described this test as follows: to each of several serial dilutions of the serum in physiological saline is added one drop of a suspension of antigen-sensitized bentonite. After mixing, a portion is transferred to a slide for microscopic examination for flocculation. A known positive serum and a negative control are tested simultaneously. The test is graded 4+ if all the particles are clumped into separate flocs, 3+ if three quarters are clumped, 2+ if half, 1+ if one quarter and 0 if non-clumped. Only 4+ and 3+ are considered positive while 2+ or less are considered negative.

The polystyrene latex test was first employed by Singer and Plotz (1956) for the diagnosis of rheumatoid arthritis. Fischman (1960) adapted it to the diagnosis of hydatid disease. Sera are cleared by centrifugation, if necessary. The most concentrated dilution of sera is 1:4 with each successive dilution having double the volume of saline. The same volume of a suspension of antigen-sensitized latex is added to each dilution. The tubes of the mixtures are incubated at 37° C for 90 minutes and then kept overnight at 4° C. The next day the tubes are centrifuged and read by the unaided eye. A known positive serum and saline control are tested concurrently. A heavy deposit indicates a positive test. Tapping the tube produces suspended flocs that are easily visible. The test is considered strongly positive when the supernatant liquid is clear and the resuspended flocs large. A weak test occurs if the supernatant liquid is slightly hazy and the flocs smaller. A negative test is opaque in appearance, either without deposit

or with a small deposit easily dispersed by tapping. Occasionally, tests appear doubtful if graded on the appearance of the deposit only. These are considered negative if on resuspension no flocs are visible to the unaided eye.

Hemolysis of red blood cells by serum in a test tube was first reported by Bordet in 1898, although Landois in 1876 had previously observed natural hemolysis by serum. In 1901, Bordet and Gengou performed the first complement fixation test to settle an argument with Ehrlich about the unity or multiplicity of complement. In the same year Widal and Lesourd applied complement fixation for the diagnosis of typhoid and tuberculosis. Later, Wasserman, Neisser and Bruch published the results of their work using complement fixation for the diagnosis of syphilis in monkeys. In the period 1905-1906, Muller and Openheimer applied the complement fixation test for gonorrhoea and Ghedini adapted it for hydatid disease in 1906 (see Kolmer, 1928).

Complement fixation has been defined as the fixation, adsorption or inactivation of complement by an interaction between an antigen and an antibody. When a complement is fixed in this manner it is not available for the lysis of other erythrocyte-antibody systems. The complement fixation test depends upon two reactions. First, it involves an antigen-antibody system plus a fixed amount of previously titrated complement. If the antigen and antibody are specific for one another, they will combine and this combination will fix the complement. The second reaction is testing for the free complement. This is accomplished

by the addition of erythrocytes sensitized by specific hemolysin. If the complement is fixed by the antigen-antibody system then no complement will be available for the lysis of the sensitized erythrocytes. If the antigen-antibody system is not specific or if one of the components is missing, then the complement remains free to produce hemolysis of erythrocytes. Therefore, a positive complement fixation test gives no hemolysis and, conversely, a negative test gives hemolysis. If either the antigen or antibody alone bind the complement they are not suited for the complement fixation test and are called anti-complementary. In order to overcome this objection two controls must be carried out with the test, a serum control and an antigen control.

The indirect hemagglutination test was introduced by Boyden (1951). He was the first to use tannic acid-treated sheep erythrocytes for the adsorption of certain proteins suspended in saline. This treatment of sheep erythrocytes with tannic acid brought about a change in the surface properties of these cells, rendering them capable of adsorbing certain proteins.

The fact that tannic acid agglutinates erythrocytes has been known for many years. Reiner and Fischer (1929) discussed the mechanism of this effect suggesting that tannic acid alters the surface properties of the erythrocytes, changing them from a hydrophilic to a hydrophobic state. Freund (1929) suggested that tannic acid brings about a change in the surface potential of the cells, which in the presence of certain electrolytes results in their agglutination. Thus, when a

specific antigen is adsorbed by the tannic acid-treated cells the erythrocytes are sensitized and, when exposed to specific immune serum, hemagglutination occurs. Ingraham (1958), McKenna (1957) and Csizmas (1960) used the same general procedure but treated the cells with formalin.

The indirect hemagglutination test was adapted by Garabedian et al (1957a) to use human group O, Rh negative blood for the diagnosis of hydatid disease. Allain and Kagan (1961) reported the further advantages of using formalized sheep cells, sensitized with tannic acid and coated with Echinococcus antigen instead of using fresh cells in the hemagglutination test. This allowed the formalized, antigen-coated cells to be stored at 5°C for two weeks and still retain their stability. These cells could be stored for periods up to 6 months if the temperature were lowered to -70°C or if they were lyophilized and stored at -20°C.

F. Specificity and Sensitivity of Diagnostic Tests for Hydatid Disease

For the complement fixation test, Fairley (1922) found that hydatid cyst fluid from sheep lung or liver cysts with high scolex content was an effective antigen, while fluid from some human cysts which possessed fewer scolices proved to be a poor antigen. Fluids obtained from non-fertile cysts which did not contain any scolices did not have any antigenic property for this test. Saline and alcoholic extracts of living or degenerating daughter cysts had no antigenic properties, whereas both alcoholic and saline extracts of scolices were potent antigen. Although alcoholic extracts of the cyst fluid were antigenic, Fairley did not find them as potent as the original fluid.

Hydatid cyst fluids from fertile cysts of cattle liver and lungs were filtered through cotton and gauze and used as antigens by Garabedian et al (1957a). The fluid was considered strongly antigenic in the complement fixation test if ^{it} showed a dilution titer of 1:2 or 1:4, or if in the indirect hemagglutination method it had a titer of 1:2. Garabedian et al (1957b) used such fertile hydatid cyst fluids stored at - 20°C in sealed ampules for a comparison of the efficacy and the complement fixation and indirect hemagglutination tests. Using sera from 16 infected persons they reported positive results in 81.75 per cent of the tests using indirect hemagglutination and 68.75 per cent with the complement fixation test. Serum from 34 people not infected with hydatid disease produced consistently negative results with the indirect hemagglutination and 5.8 per cent positive results with the complement fixation test. On the basis of these results the authors concluded

that the indirect hemagglutination test is more sensitive and more specific for the diagnosis of hydatid disease than is the complement fixation method. It was also shown to be less expensive and easier to perform.

In further work, Garabedian et al (1959) used fertile hydatid cyst fluids from cattle to compare and correlate the indirect hemagglutination test, the complement fixation test and the intradermal test for hydatid disease. Of 79 patients having hydatid disease 61 gave positive tests with complement fixation at titers of 1:2 - 1:512 for 77.2 per cent accuracy; 69 gave positive tests with indirect hemagglutination at titers of 1:10 - 1:163,840 for 87.3 per cent accuracy; and 70 gave positive tests with intradermal tests for 88.6 per cent accuracy.

Knierim and Niedmann (1962) compared the complement fixation and indirect hemagglutination tests using fertile cyst fluids from sheep and human cysts. They found that, of 203 patients with hydatid disease, the indirect hemagglutination test was positive in 171 patients while the complement fixation was positive for 151 patients. From this they concluded that the indirect hemagglutination test was the more sensitive and should be used therefore as a routine hospital test.

Fischman (1960) compared several of the tests used for detecting hydatid disease. He used fertile cyst fluids from sheep cysts in the complement fixation, latex and bentonite flocculation tests. In comparing the complement fixation and the latex tests 29 cases of known

hydatid disease were used. Both tests gave positive results in 27 cases for an accuracy of 93.1 per cent. In comparing the complement fixation and bentonite flocculation ^{tests,} sera of 33 known cases of hydatid disease were used. Both tests gave positive results in 30 cases for an accuracy of 90.0 per cent. The titers ranged from 1:8 to 1:256 for the latex test, 1:4 to 1:128 for the bentonite flocculation test and 1:10 - 1:60 for the complement fixation test.

Kagan et al (1959) using pig hydatid cyst fluid that was dialyzed in running water and concentrated three fold by pervaporation also compared several tests for hydatid disease diagnosis. On 30 known hydatid disease sera, the hemagglutination test was 96.6 per cent sensitive at titers of 1:20 - 1:25,600 and the bentonite flocculation test 90 per cent sensitive at titers of 1:5 - 1:2,560. Complement fixation gave only 36.3 per cent positive reactions. Of 246 non- Echinococcus sera tested, 14.2 per cent gave positive results with the indirect hemagglutination test at titers of 1:50 - 1:100 while 10 per cent were positive using the flocculation test.

Norman et al (1959) used two antigens in the bentonite flocculation test. Scolex antigen was prepared by saline extracts of ether-extracted, dried "hydatid sand". A hydatid cyst fluid antigen was concentrated from pig, human and horse cysts, of 413 "symptomatic" cases compared, the bentonite flocculation test gave 42 positive at titers of 1:5 - 1:2,560 for a total of 10.1 per cent and the complement fixation test gave 20 positive at titers of 1:4 - 1:128 for a total of 4.9 per cent. In 18 surgically proven cases of hydatid disease, the bentonite

flocculation test gave 18 positive at titers of 1:10 - 1:2,560 while complement fixation gave 7 positive at titers of 1:4 - 1:128. Two hundred seventy sera from individuals possibly exposed to hydatid disease were used to compare the sensitivity of the bentonite flocculation test using scolex antigen and concentrated whole cyst fluid of porcine, human and horse origin. The sensitivity was 41.9 per cent for scolex antigen, 9.6 per cent for porcine fluid antigen, 5.2 per cent for horse fluid antigen and 4 per cent for human fluid antigen.

Kagan et al (1960a) used antisera produced by immunizing rabbits with liver extracts from cotton rats, hamsters, pigs and man. The rabbit sera produced were shown to cross-react in the bentonite flocculation and indirect hemagglutination tests with hydatid cyst fluid and cyst membrane antigens of Echinococcus granulosus from pig liver. Also the tests cross-reacted with hydatid fluid and cyst antigen of Echinococcus multilocularis from cotton rat liver. The activity was stronger with Echinococcus multilocularis than with Echinococcus granulosus and occurred more frequently in the indirect hemagglutination test than in the bentonite flocculation test. The authors concluded from this that hydatid cyst fluid contains antigenic substances of host origin. In addition human sera from liver cirrhosis, nephrosis, multiple myeloma cross-reacted with liver hydatid cyst fluid antigen in both the indirect hemagglutination and bentonite flocculation tests. This reaction was considered by the author to be due to auto-antibodies stimulated by liver degeneration products.

Kagan and Norman (1961) analyzed various crude hydatid antigens by the agar double diffusion technique in micro tubes and plates. Hydatid cyst fluid, cyst membrane and scolex antigens reacted with the homologous anti sera prepared in rabbit showed at least 23 precipitin bands, 4 of which were of parasitic origin and 6 of host origin. Echinococcus multilocularis showed 27 bands of which 4 were of parasitic origin and 7 of host origin. Although scolex antigens were composed almost entirely of parasitic antigenic components, these antigens were not the most reactive in the serological tests. Hydatid cyst fluid was proved to be the most reactive in diagnostic serological tests. The authors speculated that the host antibody response in hydatid disease is against excretory-secretory products of the parasitic rather than against somatic antigens of the parasite.

Bensted and Atkinson (1953) using as an antigen fluids from sheep hydatid cysts preserved in 1:10,000 merthiolate solution tested 49 patients of which 20 were non-hydatid. Both the complement fixation and intradermal reactions were negative in these 20 cases. Of the 29 verified hydatid cases, 27 ^{were positive} by the intradermal reaction. The results of these comparative tests are given in Table I.

Table I. Comparisons of Sensitivity of Serological and Skin Tests in the Diagnosis of Hydatid Disease

| Reference | Antigen | Diagnostic test | Number of cases | Positive reactions | Titers |
|-----------------------------|--|------------------------------|------------------------------------|------------------------------|---|
| Fairley (1922) | Alcohol and saline extraction of scolices and membranes | C.F. | 83 | 83.3% | - |
| Garabedian et al (1957b) | Aspirated, filtered fertile hydatid fluid from cysts of cattle liver and lungs | I.H. C.F. | 16 " | 81.25% 68.75% | - |
| Garabedian et al (1959) | Fertile cyst fluid from hydatid cysts of cattle | I.H. C.F. I.D. | 79 " " | 87.3% 77.2% 88.6% | 1:10-1:163,840 1:2 - 1:512 |
| Knierim and Niedman (1962) | Fertile cyst fluid from sheep and human cysts | I.H. C.F. | 203 " | 84% 74% | - - |
| Fischman (1960) | Fertile cyst fluid from sheep cyst | C.F. L.F. C.F. B.F. | 29 " 33 " | 93.1% " 90.9% " | 1:10 - 1:60 1:8 - 1:256 1:4 - 1:128 |
| Kagan et al (1959) | Hydatid fluid from pig cysts dialyzed in running water and concentrated by pervaporation | B.F. I.H. | 30 " | 90% 96.6% | 1:5 - 1:2,560 1:20- 1:25,600 |
| Norman et al (1959) | Scolex antigen by saline extracts of ether-extracted, dried hydatid sand hydatid fluid antigen concentrated from hog, human, horse cysts | B.F. C.F. C.F. B.F. | 413 symptomatic 18 proven | 10.1% 4.2% 39% 100% | 1:5 - 1:2,560 1:4 - 1:128 1:4 - 1:128 1:10- 1:2560 |
| Bensted and Atkinson (1953) | Fertile cyst fluid from sheep | C.F. I.D. | 29 29 | 93.2% 86.4% | - |

C.F. = Complement fixation
 L.F. = Latex flocculation
 I.H. = Indirect hemagglutination
 I.D. = Intradermal
 B.F. = Bentonite flocculation

G. Antigen Preparation For the Sero-Diagnosis of Hydatid Disease

Serious investigators have attempted to isolate the specific chemicals agents in hydatid material which are responsible for the formation of the antibodies demonstrable by these various tests. Fairley (1922) used the powdered membranes of hydatid cysts extracted for several days with absolute alcohol and powdered scolices extracted with either absolute alcohol or saline solution as antigens in the complement fixation test. He found them all to be potent. Miles (1927) used alcoholic extracts of hydatid scolices and found they were more potent than hydatid cyst fluid in the complement fixation test, precipitation test and Casoni test. He believed that the antigenic factor was alcohol-soluble rather than saline-or acetone-soluble and thought it was mainly lipoidal in nature.

In later work, Senekji (1941) isolated a "polysaccharide" by extracting scolices with four volumes of acetone overnight. The acetone fraction when dried gave a powder which was active using the Casoni test and precipitin test. Another active preparation was made by extracting the scolices for 48 hours with N/4 trichloroacetic acid. The supernatant liquid was neutralized with NaOH and the precipitate separated, washed with absolute alcohol, washed with ether, dried and used in the same tests for hydatid disease. This antigen was soluble in acids, precipitated in alkaline solutions, gave a strong Molisch test for carbohydrates but did not react with Biuret's or Millon's test for proteins. It reduced Benedict's solution only after hydrolysis.

Culbertson and Rose (1941) derived antigens suitable for eliciting skin reactions in patients with hydatid disease from several different cestodes, including larval stages of Echinococcus granulosus, Taenia saginata, Taenia crassicolis, Hymenolepis cesticillus and in both the adult and sparganum of Diphyllobothrium mansonioides. The active substances were saline extracts prepared from dried, ground cestodes. In addition to the whole worm, a protein-free "polysaccharide" derived from Taenia crassicolis was prepared by Culbertson and Rose after the method of Campbell (1939). In this procedure an alcoholic precipitate was prepared in acetic acid solution. The absence of proteins was shown by negative Biuret test, no precipitate with phosphotungstic acid and no nitrogen by Nesslerization of the hydrolysate. This "polysaccharide" extract elicited a skin reaction even when the stock solution, which contained 10 mgm/0.1 cc was diluted a hundred times.

Pirosky et al (1941) used cyst fluid, membranes and "hydatid sand" to extract a "polysaccharide" antigen. The fluid was filtered through a bacterial filter, dialyzed and the proteins precipitated with saturated NaCl. The remaining liquid which did not contain any trichloroacetic precipitable materials or heat coagulatable substances, was treated with alcohol to obtain the antigen. The membranes were extracted with saline for 48 hours in the cold, freed from protein by precipitation and the supernatant liquid used. The "hydatid sand" was centrifuged, washed and treated in the same manner

as the membranes. This antigen was an amorphous white powder, insoluble in water and organic solvents. It gave a negative test with Fehling's, Biuret or iodine solutions. It contained 2.55 per cent nitrogen and hydrolysis with concentrated HCl gave the reaction of levulinic acid while hydrolysis with 3 per cent HCl for three hours gave a reducing sugar which with phenylhydrazine gave a glucosone. This antigen was active in the Casoni intradermal test. Complement-fixing antibodies were demonstrated when this polysaccharide was injected subcutaneously into rabbits, but in humans having hydatid disease it was uncertain that complement-fixing antibodies against this polysaccharide were present.

Čmelik (1952b) extracted a "polysaccharide" antigen from the membranes of pig liver cysts using four different extraction procedures; extraction with water, extraction with trichloroacetic acid, enzymatic hydrolysis with pepsin and a combined hydrolysis with pepsin and trypsin. The extraction with water involved adjusting the pH to 3.5 with acetic acid and dialyzing. Alcohol was added to the solution to give an alcohol concentration of 75 percent and the precipitate washed and dried. In the second method, the fresh membranes were extracted with five per cent trichloroacetic acid for 24 hours in the cold. After centrifuging, the solution was dialyzed and acetone added to an acetone concentration of 70 per cent. The resulting precipitate was purified by reprecipitating, washed and dried. For the pepsin digestion, the membranes were ground in water and the pH adjusted to 4.0 using normal HCl. After 24 hours incubation at 37°C, the

solution was dialyzed and the procedure with acetone followed as in the trichloroacetic acid extraction.

In the combined digestion with pepsin and trypsin the incubation was first carried out at pH 3.5 and then again at pH 7.8. After filtering through a glass filter a "polysaccharide" was obtained. It is of interest to note that Kilejian et al (1962) followed this latter procedure in an attempt to isolate polysaccharides from the laminated membrane, but gross bacterial growth at the neutral pH for the trypsin digestion made the procedure impractical in their hands.

The presence of protein substances and amino acids in these products was tested for by Čmelik using sulphosalicylic acid, Biuret's reaction and ninhydrine, while for the detection of sugar the reaction of Molisch was employed. The presence of desoxypentoses was proven by the reaction of Dische. The reactions for pentoses were followed after Tollens and Bial and for ketoses after Seliwanoff. Glucosamine was proven to be in the hydrolysate using the procedure of Elson and Morgan. The results of these tests are summarized in Table II for the various extracts.

The complement fixation and intradermal tests were used by Čmelik (1952b) to determine the activity of these various extracts. The "purest polysaccharide" obtained by combined digestion with pepsin and trypsin did not fix complement in guinea-pigs sensitized with the material. On the other hand, this product gave a temporary and a constant positive skin-reaction in Echinococcus carriers.

Table II. Chemical Reactions of Products Obtained by Extraction of Hydatid Cyst Membranes (After Čmelik, 1952b)

| Extracts | Sulpho-salicylic acid | Ninhydrine | Biuret | Molisch | Dische | Tollens | Bial | Seliw-anoff | Elson Morgan |
|----------------------|-----------------------|------------|--------|---------|--------|---------|------|-------------|--------------|
| Water | + | + | + - | ++ | - | - | - | - | ++ |
| Trichloroacetic acid | - | - | - | ++ | - | - | - | - | ++ |
| Pepsin | + | + - | - | ++ | ++ | - | - | - | ++ |
| Pepsin and trypsin | - | - | - | ++ | ++ | - | - | - | ++ |

(++) indicates strongly positive,

(+) positive,

(+ -) weakly positive,

(-) negative.

Another worker, Magath (1959), reported that the antigenic property of hydatid cyst fluid was in the protein-free, acetone-ether insoluble portion using the complement-fixation test, but he noted that this active fraction moved electrophoretically with gamma globulin like proteins of cyst fluid. It could, however, be separated from the proteins by protein precipitation and still retain its activity.

MacNab (1948) prepared an active "polysaccharide" fraction which he used as an antigen in the precipitin test for a patient having a pulmonary hydatid cyst. No method of preparation was indicated.

Pirosky et al (1949) used various preparations of as antigens in the complement fixation test. The 4 materials used: (1) the hydatid cyst fluid, (2) water soluble fractions of hydatid tissues, (3) organic solvent soluble fractions of hydatid tissues and (4) a so-called "antigenic integral". This latter was a suspension in physiological saline of cystic membranes. It contained one per cent organic substances. Cysts from cattle liver were used as the source for all the material.

The water soluble material was separated into different fractions containing proteins and polysaccharide. The protein from the fluid was precipitated with saturated sodium chloride at pH 3.2, dialyzed, centrifuged and redissolved. This process was repeated several times and the product was finally dissolved in physiological saline at pH 7.6. This fraction contained 2.427 grams nitrogen per 1000 grams of protein. The scolices were ground in a Waring Blendor and a method similar to that for the fluid followed. The final precipitate contained 0.5 grams of nitrogen per 1000 grams of protein. The cystic membranes

were first ground in a Waring Blender container previously chilled to -40°C . This was followed by extraction in sterile distilled water for 48 hours. The resulting solution was filtered and the liquid subjected to essentially the same procedure as that for hydatid fluid. The product was a protein containing 4.35 grams of nitrogen per 1000 grams of protein.

The deproteinized liquid from the hydatid cyst fluid, membranes and scolices was treated with 5 percent trichloroacetic acid for 24 hours, centrifuged, made acid to pH 5.5 and an equal volume of 96 per cent alcohol in a buffered solution added. Saturated sodium chloride was added and a precipitate formed. The polysaccharide was dissolved and reprecipitated with alcohol. It gave a negative test with Biuret, a strongly positive Molisch test, negative Seliwanoff and a positive test with levulinic acid. It contained 2.55 per cent nitrogen. The organic solvent fractions were prepared by using alcohol, acetone, ether and various combinations of these as extracting agents. These authors found that the complement fixation property depended on the protein content. Of the water soluble material, only the protein part reacted with the corresponding antibody. This protein part and the hydatid cyst fluid were species-specific when tested against sera from patients with hydatid disease, Taenia saginata infection and several bacterial infections. In the organic soluble material group specificity was observed for taeniid tapeworms at certain concentrations. The hydatid antigeno integral was capable of fixing complement but no definite conclusions could be drawn.

The author concluded that the protein fraction- by specificity and sensitivity- was the most appropriate antigen for complement fixation test.

Dennis (1937) precipitated the "protein" from hydatid cyst fluids of livers and lungs of cattle and sheep using 5 per cent trichloroacetic acid. He found that 3 per cent trichloroacetic acid did not give a precipitation of all the active material and that 8 per cent gave little increase. The precipitated protein was washed and redissolved in physiological saline, reprecipitated, washed and again dissolved in physiological saline and finally subjected to Seitz filtration. This material was shown to be active using the complement fixation test, the precipitin test and the Casoni test for hydatid disease.

Pautrizel and Sarreau (1947) prepared three different products from hydatid scolices and all were found to be good antigens when tested intradermally. These fractions were: a glycolipid complex prepared by trichloroacetic acid precipitation, a glycoprotein-complex made by precipitation with absolute alcohol and ether and a polysaccharide fraction prepared by precipitating the proteins with phosphotungstic acid and then precipitating the supernatant with absolute alcohol.

Norman et al (1959) obtained three materials from "hydatid sand". For one the "sand" was extracted with physiological saline for 24 hours in the cold. In another, lyophilized "sand" was used and for the third product a fat-free antigen was prepared by extracting the lyophilized "sand" with ether. All three antigens were shown to be active using the bentonite flocculation test and the complement fixation test.

Kagan et al (1960b) in studying the best antigen in the indirect hemagglutination test, used hydatid cyst fluid and saline extracts of dried scolices and cyst membranes of Echinococcus granulosus obtained from pig liver cysts. Also, they used "fluid", scolices and whole cyst material from Echinococcus multilocularis of experimentally infected cotton rats and gerbils. These crude antigens were tested with specific antisera prepared in rabbits against each material and also against sera from individuals with previous hydatid disease. Cross-reactions between various antigens and antisera were obtained when tests were made with antisera of immunized rabbits. These cross-reactions were more evident in cyst and scolex antigens of both species than in hydatid fluid or membrane antigens.

Hydatid cyst fluid was the best antigen with sera from patients with Echinococcus granulosus. Trichloroacetic acid was used to precipitate "fluid" obtained from Echinococcus multilocularis cysts as well as from Echinococcus granulosus. The Echinococcus granulosus fractions had no advantage over crude hydatid cyst fluid, whereas fractions of Echinococcus multilocularis were superior to the unfractionated material in the serological test. They suggested that a more active antigen might be obtained if a method were found for extracting the lipo-protein-polysaccharide complexes and that the active antigenic component in the indirect hemagglutination test might be a protein.

Bono and Pellegrini (1956) separated hydatid cyst fluid proteins electrophoretically. When cattle origin and sheep origin fractions

were compared, they showed little variation in their electrophoretic patterns. However, the fluid proteins from cysts from different organ locations gave slightly different rates of migration. The proteins separated all moved toward the anode and some had different rates of migration depending on their origin in fertile or unfertile cysts.

Protein extracts of the laminated membrane and germinative layer of echinococcal cysts from the liver and lungs of cattle were also electrophoretically fractionated by Morellini and Ferri (1960). The extracts were prepared by mechanically triturating the material at -40°C in a special apparatus. After grinding, it was shaken at 4°C in a cold room for 24 hours, centrifuged and the supernatant removed. Upon lyophilization of the supernatant liquid a white water-soluble precipitate was obtained and this material used. They found four proteins that migrated differently and, of these, only the center two proteins possessed antigenic activity using the complement fixation test. These rather pure extracts, when injected into rabbits, produced antibodies which were specific against that antigen and did not give any cross-reaction against antigens prepared from other species as evidenced by the complement fixation test. They estimated that the membranes contained about 6,000 times more of the antigenic protein for this test than did the hydatid cyst fluid.

In so far as the intradermal and complement fixation tests are concerned, these findings are summarized in Tables III and IV.

Table III. Active Antigens in the Casoni Skin Test for Hydatid Disease

| Source | Antigen | Identification | Results | Reference |
|--|--|--|---------|------------------------------|
| Hydatid cyst scolices | polysaccharide | 1. positive Molisch test 2. negative Biurets and Millon's tests 3. reduction of Benedicts after hydrolysis | active | Senekji (1941) |
| <u>Taenia crassicolis</u> | polysaccharide | 1. negative Biuret test 2. no precipitation with phosphotungstic acid 3. no nitrogen by Nesslerization of the hydrolysate | active | Culbertson and Rose (1941) |
| Hydatid cyst fluid, membranes and hydatid sand | polysaccharide | 1. negative Fehlings test 2. negative Biuret and iodine tests 3. hydrolysis with conc. HCl gave a reaction with levulinic acid 4. hydrolysis for 3 hours gave a reducing sugar which gave a glucosone with phenylhydrazine | active | Pirosky (1941) |
| laminated membranes of hydatid cysts | polysaccharide | 1. negative sulfosalicylic acid test 2. negative ninhydrin test 3. negative Biuret test 4. positive Molisch test 5. positive Dische test 6. negative Follen's test 7. negative Bial's test 8. negative Seliwanoff test 9. positive Elson Morgan test | active | Čmelik (1952) |
| Scolices of hydatid cysts | 1.glycolipid 2.glycoprotein 3.polysaccharide | - - - | active | Fautrizel and Sarreau (1947) |
| Hydatid cyst fluid | protein | - | active | Dennis (1937) |

Table IV. Active Antigens in the Complement Fixation Test for Hydatid Disease

| Source | Antigen | Identification | Results | Reference |
|--|---|--|---|-----------------------------|
| Hydatid cyst fluid | Protein free, acetone-ether insoluble portion | - | active | Magath (1959) |
| Hydatid cyst fluid, membrane and hydatid sand | Polysaccharide | <ol style="list-style-type: none"> 1. negative Pehlings test 2. negative Biuret and iodine tests 3. hydrolysis with con. HCl gave a reaction with levulinic acid 4. hydrolysis for 3 hours gave a reducing sugar which gave a glucosone with phenylhydrazine | active only in rabbits | Pirosky (1941) |
| Laminated membranes of hydatid cysts | Polysaccharide | <ol style="list-style-type: none"> 1. negative sulfasalicylic acid test 2. negative ninhydrin test 3. negative Biuret test 4. positive Molisch test 5. positive Dische test 6. negative Tollen's test 7. negative Bial's test 8. negative Seliwanoff test 9. positive Elson Morgan test | negative in sensitized guinea pigs | Čmelik (1952) |
| Fluid, scolices and membranes of hydatid cysts | Polysaccharide | <ol style="list-style-type: none"> 1. negative Biuret test 2. positive Molisch test 3. negative Seliwanoff test 4. positive test with levulinic acid | negative | Pirosky <u>et al</u> (1949) |
| Fluid, scolices and membranes of hydatid cysts | Protein | - | active | Pirosky <u>et al</u> (1949) |
| Hydatid cyst fluid | Protein | - | active | Dennis (1937) |
| Hydatid cyst membranes | Protein | electrophoretically | positive only in the 2 center protein fractions in electrophoregram | Morellini and Ferri (1960) |
| Hydatid cyst scolices | Lipoidel | - | active | Hiles (1927) |

Summary

Any investigation into the antigenic factors responsible for the production of antibodies in hydatid disease which are detectable by the various serological tests available is thus faced with a considerable literature that is in many respects confusing and often conflicting. Although no attempt has been made here to review in detail the literature on hydatid chemistry per se, it is safe to say that the chemical compositions of hydatid cyst fluid and tissues are not, even now, very well known. Much of the existing confusion in hydatid immunochemistry comes from different methods of testing for antigenic activity by different authors and also from the many different methods which have been followed in preparing the various antigenic products and extracts used.

The purpose of the present study was to observe, under conditions as nearly standard as possible, various biological, physical and chemical properties of the antigenic material which is active in the indirect hemagglutination test for hydatid disease.

MATERIALS AND METHODS

A. Indirect Hemagglutination Test as Applied to Hydatid Disease

The indirect hemagglutination method described by Garabedian et al (1957a) was followed throughout this study with the addition of a block titration method for the antigens studied. Four dilutions of antigen were used in each experiment. With the exception of the antigen, all other variables in the antigen-antibody system were kept constant. Thus the tannic acid used was from a single batch of type 33 fluffy Dyewood Brand, Glasgow, Scotland. The saline-phosphate buffer was Bacto-streptolysin O buffer (code 0516) from Difco Laboratories, Detroit, Michigan. All other factors were also kept constant throughout the experiments as indicated below. These factors included the human positive antiserum, the human type O Ph⁺ erythrocytes and the serum diluents.

1. The Standard Antiserum

The serum used throughout these experiments was taken from a pool of sera of 11 different hydatid disease patients. Approximately four-fifth of this pool was contributed by one woman and was collected by the Department of Tropical Health for the World Health Organization to be used as an International Standard antiserum for the diagnosis of hydatid disease. The titer of this standard serum was 1:5,120 plus or minus one serial dilution when used against highly fertile hydatid cyst fluid from sheep as the antigen.

2. Human type O Rh⁺ Erythrocytes

The donor of the cells used in these experiments was a non-smoking, non-drinking individual 45 years of age who had previously been tested by the indirect hemagglutination test and found to be negative for hydatid disease. Forty cc of blood were collected weekly following a 12 hour fast and divided into two equal portions. One portion was allowed to clot and the serum removed and stored in the frozen state for use as a diluent. The other portion was put in modified Alsever's solution and stored at 4°C until used.¹

The one variation in this procedure occurred during a few of the late experiments when, due to the anemic condition of the donor, additional blood samples were obtained from a similar male donor whose blood was of the same type. These cells were compared in duplicate experiments with those of the principal donor without any change in results, standardized against a standard hydatid cyst fluid and therefore it may be safely assumed that this substitution had no effect on the results obtained. Each substitution is indicated in the results, however.

¹Modified Alsever's solution:

| | |
|----------------------|-------------|
| Dextrose | 20.50 grams |
| Sodium citrate | 8.00 grams |
| Sodium chloride | 4.20 grams |
| Citric acid | 0.55 grams |
| Distilled water q.s. | 1000 ml |

3. Hydatid Cyst Fluids for Comparisons of Antigen Source

The fluids from individual hydatid cysts were aseptically collected by syringe in 100 ml vials and immediately frozen and stored until use at -20°C .

a. Human Cyst Fluids: These fluids were collected from fertile hydatid cysts of the brain, thigh and peritoneal cavity, each from a different patient in the American University Hospital. They were collected during the month of April 1963 and used in August 1963.

b. Sheep and Cattle Cyst Fluids: These individual fluids were collected on May 7, 1963 from five cysts of sheep liver, five of cattle liver, three sheep lungs and one of cattle lung. These animals were slaughtered in the Beirut Municipal Abattoir. All cysts were highly fertile. These fluids were tested in July 1963.

c. Burro Cyst Fluids: These fluids were collected on March 6 and October 2, 1963 from nine fertile cysts in the livers of animals purchased from farmers and slaughtered at the American University farm. These fluids were tested in July and October 1963.

d. Camel Cyst Fluids: These fluids were collected on July 5, 1963, and March 4, 1964 from eight fertile cysts in the lungs of animals slaughtered in the Municipal Abattoir of Damascus, Syria. These fluids were tested in August 1963 and April 1964.

e. Gerbil Cyst Fluids: These fluids were collected in October 1963 from three fertile secondary hydatid cysts from three ^{of} gerbils (Gerbillus gerbillus). Sheep were the original scolex source. All three cysts were from the peritoneal cavity and were tested in October 1963.

4. Taenia Hydatigena Cysts

These fluids were collected in August 1963 from six Taenia hydatigena cysts from the peritoneal cavities of sheep slaughtered in the Beirut Municipal Abattoir. The fluids were tested in August 1963.

B. Fractions of Cystic Fluids

Fractions of Echinococcus granulosus hydatid cyst fluid were prepared by Mr. M. Koussa following the procedure described in et al Milejian/(1962). The fractions of Taenia hydatigena cyst fluid were prepared similarly by Mr. M. Koussa. These materials had been lyophilized and stored at -20°C .

For extraction, 20 milligrams of each of the lyophilized fractions was taken. These fractions were the freezing-thawing precipitate, the dialysis precipitate, the trichloroacetic acid precipitate, and the alcohol precipitate. Each was extracted with five cc of 0.9 per cent saline after homogenization in a Teflon-glass tissue grinder then left overnight in the refrigerator at 4°C . The resulting solutions were centrifuged and the supernatant tested at different dilutions.

In another procedure, the same materials were reconstituted to their original fluid volumes using Lacto-streptolysin B buffer. The antigenic activity of the fractions by each of these procedures were then compared to that of the original cyst fluid from which they had been prepared.

Each fraction was examined by Mr. M. Koussa by infra-red spectroscopy and paper electrophoresis and, in some instances, by paper chromatography for amino acids and sugars.

C. Scolex Extracts

Scolex extracts were prepared from hydatid cyst fluids of both cattle and sheep using the procedure of Kagan et al (1950b). The only modification was that instead of diluting the extract 1:400 as Kagan did, the extract was diluted only 1:40.

The method of Hess and Deutsch (1948) for the fractionation of the sera of cattle was applied to the fractionation of bovine hydatid cyst fluid. A volume of 3000 ml of fertile hydatid cyst fluid at pH 7.7 was cooled to 0°C and 1685 cc of 50 per cent ethanol which had previously been cooled to -20°C was added to produce a final ethanol concentration of 18 per cent. The resulting precipitate was centrifuged and lyophilized to yield 213 mg of dry material. Twenty mg of this precipitate was extracted with 5 cc saline overnight in the refrigerator at 4°C. and centrifuged. The supernatant liquid was made up to 1:10 and 1:20 dilutions using Bacto-streptolysin O buffer. Aliquots of the precipitate were made up to their original volumes with Bacto-streptolysin O buffer and tested for antigenic activity. This precipitate was examined electrophoretically by Mr. M. Koussea.

Another scolex-fluid antigen was prepared by adding 2 cc. of packed scolices to 30 cc of fertile hydatid cyst fluid. This fluid 1:8 tested originally for a serum titer of 1:5120 and an antigen titer of 1:8. The scolex cyst fluid mixture was homogenized using a Teflon-glass tissue grinder. The fluid containing lysed scolices was left overnight in the refrigerator at 4°C, centrifuged and the supernatant liquid frozen until used. In a replication of this procedure the activity of the scolex-fluid antigen was determined before freezing.

RESULTS

A. Comparison for Antigenic Activity of Different Hydatid Cysts from the Same Organ of the Same Animal

The indirect hemagglutination test using standard human antiserum was performed on highly fertile hydatid cyst fluids. A sample was taken from each of five different cysts of the liver of one sheep and each of five different cysts of the liver of one cow. The results of these tests are summarized in Table V and VI.

From these tables it can be seen that there was no appreciable difference in the serum titers obtained or in the antigenic concentration of hydatid cyst fluids from highly fertile cysts of the same organ of the same animal.

B. Comparison for Antigenic Activity of Hydatid Cyst Fluid from Various Animals

A further comparison was done to see if the antigen involved in the indirect hemagglutination test, against the standard human antiserum, differed in hydatid cyst fluids taken from different species of host animals. These results are summarized in Table VII and shown graphically in Figure I.

Table V. Antibody Titers Obtained from Five Cysts of One Sheep

| Experiment No. | Antigen Dilutions | | | | |
|----------------|-------------------|---------|--------|--------|------|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| I | 1:5120 | 1:10240 | 1:5120 | 1:5160 | 1:80 |
| II | 1:5120 | 1:5120 | 1:5120 | 1:6680 | - |
| III | 1:5120 | 1:5120 | 1:5120 | 1:4470 | - |
| IV | 1:5120 | 1:5120 | 1:2560 | 1:4370 | - |
| V | 1:5120 | 1:10240 | 1:5120 | 1:5120 | - |

Table VI. Antibody Titers Obtained From Five Cysts of One Cow

| Experiment No. | Antigen Dilutions | | | | |
|----------------|-------------------|---------|--------|--------|------|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| I | 1:5120 | 1:5120 | 1:5120 | 1:1280 | - |
| II | 1:10240 | 1:10240 | 1:5120 | 1:1280 | - |
| III | 1:5120 | 1:10240 | 1:5120 | 1:1280 | - |
| IV | 1:5120 | 1:5120 | 1:5120 | 1:1280 | - |
| V | 1:5120 | 1:10240 | 1:5120 | 1:1280 | - |

| | | | | | | |
|--|-----------------------|-----------------------|-----------------------------|-----------------------------|------------------------------------|---|
| bil itoneal ity ysts rtille) | - | 9 $\bar{x} = 9$ | 9 9.4 $\bar{x} = 9.1$ | 5 5.5 $\bar{x} = 4.7$ | 0 0 $\bar{x} = 0$ | - |
| le liver sts -fertile) | 0 0 6 | 0 0 6 | 0 0 6 | 0 0 0 | 0 0 0 | - |
| ia tigena sts rtille) | 9 9 9 8 8 | 9 9 9 8 8 | 8 8 8 8 8 | 8 8 8 8 8 | 8.6 8 8.2 8 7.4 7.4 | 2 |

* Fluid from a single cyst used for each experiment.

** Each value on this table which represents a maximum antigen titer is the mean of five replications.

Table VII. Comparison of Fluid from Different Animals (Expressed in Tube Number)

| Source and Number of Experiment* | Antigen Dilutions** | | | | | |
|---|---------------------|--|---|----------------------------------|----------------------------------|------|
| | undiluted | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| Sheep Liver <u>5 Cysts</u> (Fertile) | - | 9 9 \bar{x} = 9 | 10 9 \bar{x} = 9.4 | 9 9 \bar{x} = 8.8 | 9 9.4 9.8 \bar{x} = 9 | 3 |
| Sheep Lung <u>3 Cysts</u> (Fertile) | - | 9 9 \bar{x} = 9 | 9 9 \bar{x} = 9 | 9 9 \bar{x} = 9 | 4 4 \bar{x} = 3.7 | - |
| Cattle Liver <u>5 Cysts</u> (Fertile) | 9 \bar{x} = 9 | 9 10 9 \bar{x} = 9.2 | 9 10 9 \bar{x} = 9.6 | 9 9 9 \bar{x} = 9 | 7 7 7 \bar{x} = 7 | - |
| Cattle Lung <u>1 Cyst</u> (Fertile) | - | 9 | 9 | 8.9 | 4 | - |
| Burro Liver <u>9 Cysts</u> (Fertile) | - | 5.8 5.8 7.8 7.4 \bar{x} = 7.2 | 5.8 3 2 3 \bar{x} = 5.2 | 0 0 0 2 \bar{x} = 1 | 0 0 0 0 \bar{x} = 0 | - |
| Camel Lung <u>8 Cysts</u> (Fertile) | - | 7 6 7 6 \bar{x} = 6.6 | 7 6.2 6.2 3 7 \bar{x} = 6.3 | 3 2 2 0 \bar{x} = 2.6 | 0 0 0 3 \bar{x} = 0.4 | - |
| Human Hydatid Cyst Fluid <u>3 cysts</u> (Fertile) | - | 9 9 \bar{x} = 9 | 9 9 \bar{x} = 9 | 9 9 \bar{x} = 9 | 9 9 \bar{x} = 7.3 | - |

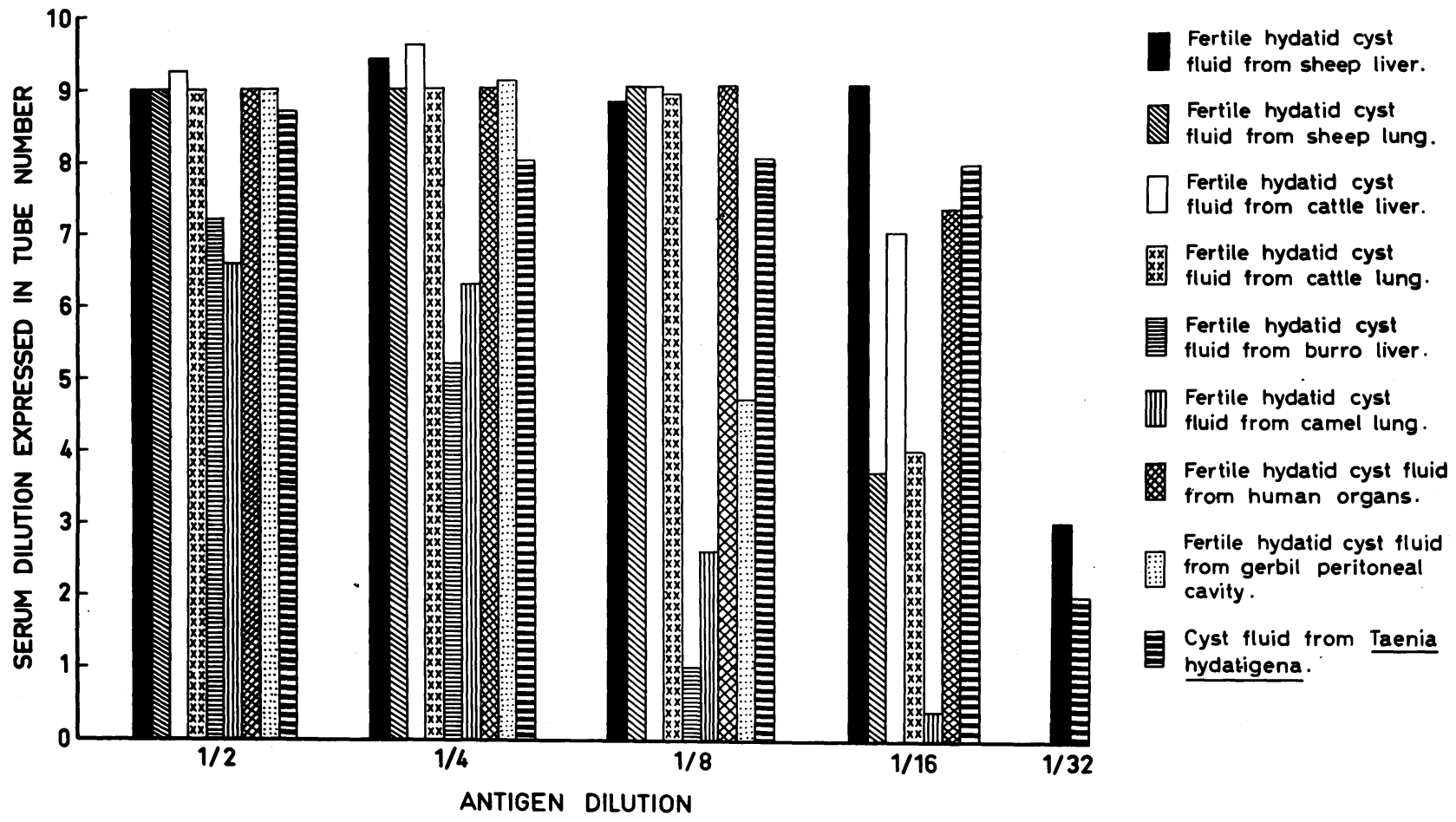


FIG. 1. COMPARISON OF FLUID FROM DIFFERENT ANIMALS (expressed in tube number).

The relationship between tube number and dilution value

is:

| | | | | | | | | | | |
|----------------|----|----|----|-----|-----|-----|-------|-------|-------|--------|
| tube number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| dilution value | 20 | 40 | 80 | 160 | 320 | 640 | 1,280 | 2,560 | 5,120 | 10,240 |

which represents a logarithmic function. The mathematical formula is:

$$Y = (10) (2)^X$$

where Y is the dilution value (i.e. 1:20, 1:1280, etc.) and X is the tube number. This can be written in logarithmic form as:

$$\log Y = X \log 2 + 1.0$$

Hence to convert a fractional tube number to dilution value, it is multiplied by 0.301 (the log of 2); 1.0 is added and the antilog found. For example:

$$\begin{aligned} &\text{for a tube number of } 8.26 \\ (8.26) (0.301) &= 2.4863 \\ 2.4863 + 1.0 &= 3.4863 \\ \text{antilog } 3.4863 &= 3.065 \end{aligned}$$

Therefore the tube number 8.26 represents a 1:3065 dilution.

Figure II. Gives the graphical relationship between tube number and dilution value. The proper log dilution value corresponding to the tube number is found. The antilog of this gives the dilution value.

A similar graph could be constructed on semi-log paper giving the dilution values directly without taking antilogs.

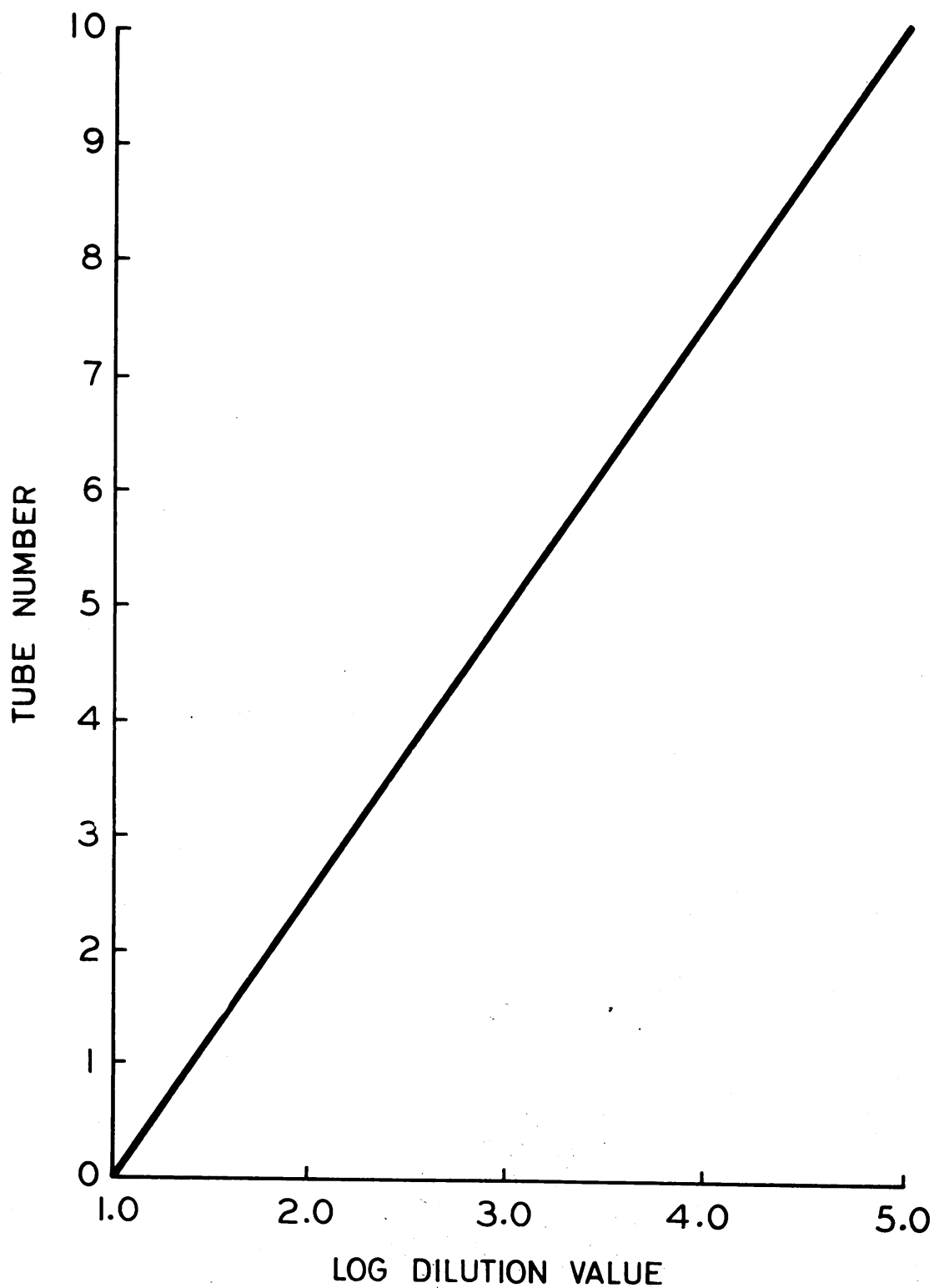


FIG. 2. RELATIONSHIP OF DILUTION VALUE TO TUBE NUMBER.

From these data it appears that the host source of antigen is of little if any significance as long as the fluid is obtained from healthy, fertile cysts. Somewhat lower serum titers were obtained with camel and burro fluids, however, than with the others. On the other hand, antigen titers differed somewhat from cyst of one host animal to another. An interesting finding was that Taenia hydatigena cyst fluid was equally useful as a source of antigen for the indirect hemagglutination test. A group specific factor is therefore evident.

Infertile hydatid cyst fluids possessed little or no antigenicity no matter what host they were obtained from. The exception was infertile cyst fluid from secondary infections in mice, where the cysts were alive but had not yet developed scolices. In one instance the full serum titer was obtained at an antigen titer of 1:4. Repeating the procedure with three other infected mice, the infertile fluid was positive at a reduced serum titer in one instance and negative in the other two (see Table VIII). These cysts were examined under the dissecting scope to insure that the hydatid cyst fluids were in fact infertile. These results indicated that hydatid cysts begin to produce the indirect hemagglutinating antigen even before scolices arise.

Table VIII. Serum Dilution Using Infertile Hydatid Cyst Fluid from Secondary Infection of Mice.

| Experiment No. | Antigen Dilution | | |
|----------------|------------------|-----------|------|
| | 1:2 | 1:4 | 1:8 |
| I | 1:5120 | * 1:10240 | 1:80 |
| II | 1:2560 | * 1:2560 | 1:80 |
| III | 0 | * 0 | 0 |
| IV | 0 | * 0 | 0 |

* Mean of three replications

C. The Effect of Temperature on the Antigen

Since Bacigalupo (1925) reported that heating hydatid cyst fluid for five minutes at 100°C did not destroy its antigenic properties in the intradermal and complement fixation tests, experiments were designed to test the temperature inactivation of hydatid cyst fluid using the indirect hemagglutination test. Known active fluid from cattle liver cysts was heated to various temperatures for different periods of time. The activity was tested after rapid cooling to room temperature. For temperatures up to 80°C ten cc of the fluid in a test tube was placed in a constant temperature water bath. For 80°C the 10 cc was divided into two equal portions and placed in the water bath as above. This smaller volume allowed the fluid temperature to reach that of the water bath sooner since time is more critical at elevated temperatures. For 100°C the fluid was divided into 1 cc portions and the same procedure followed.

The pool of fertile hydatid cyst fluid from cattle livers had an original antigen titer of 1:8 (serum titer 1:5120) when compared to the standard human antiserum. Heating this fluid to 56°C for 25 minutes and again testing the titer of the fluid against the standard human antiserum, showed that there was no decrease in the titer of the antigen or serum and hence no loss of activity. Heating it to 70°C for 25 minutes also produced no change, while heating it at 80°C for 25 minutes gave doubtful results. When time at 80°C was reduced to five minutes the titer of the antigen was reduced to 1:4. At 100°C for five minutes the fluid lost its antigenicity completely. These data are summarized in Table IX.

Table IX. The Effect of Temperature on the Antigen
(In Terms of Antibody Titer)*

| Temperature maintained for 25 minutes | Antigen Dilutions | | | | |
|---|--|--------|-----------|-------|------|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| 21°C (room temperature) | 1:5120 | 1:5120 | ** 1:5120 | 1:640 | - |
| 56°C | 1:5120 | 1:5120 | ** 1:5120 | 1:640 | - |
| 70°C | 1:5120 | 1:5120 | ** 1:5120 | 1:640 | - |
| 80°C | D O U B T F U L R E S U L T S | | | | |
| 100°C | 0 | 0 | ** 0 | - | - |
| Temperature maintained for 5 minutes | | | | | |
| 80°C | 1:5120 | 1:5120 | ** 1:320 | - | - |
| 100°C | 0 | 0 | ** 0 | - | - |

* All experiments repeated two times with identical results.

** Mean of three replications.

D. The Effect of Filtration on the Antigenicity of Hydatid Cyst Fluids

One hundred cc of fertile hydatid cyst fluid from sheep livers, whose original antigen titer was 1:8 (serum titer 1:5120) when compared to the standard human antiserum, was filtered through a Seitz filter of one micron porosity. The filtrate was tested again to determine any change in the antigen titer, and the filtrate was found to have lost its activity completely. The same Seitz filter pad was then placed in a flask containing 100 cc 0.9 per cent saline solution and kept in the refrigerator overnight with occasional shaking. It was then centrifuged and the saline tested by the indirect hemagglutination test. This gave entirely negative results.

This experiment was repeated by taking 80 cc of fertile hydatid cyst fluid from sheep livers which also had an antigen titer of 1:8 in the indirect hemagglutination test when compared to the standard human antiserum. This fluid was filtered through a Seitz filter of the same porosity and again the antigenicity was lost. The Seitz filter paper was returned this time to the fluid filtrate and left with occasional shaking overnight in the refrigerator. It was then centrifuged and the supernatant tested. Again the results were negative.

A further experiment was performed where a new Seitz filter pad was added to a flask containing the same fertile hydatid cyst fluid. The fluid and filter pad were allowed to stand overnight in the refrigerator with occasional shaking and then centrifuged. The supernatant liquid was tested using the indirect hemagglutination test and the results were negative. This proved that the antigenic activity was lost by adsorption by the filter pad and not by filtration (See table X).

Table X. The Effect of Seitz Filtration on the Antigenicity of Hydatid Cyst Fluid* (In Terms of Antibody Titer).

| Operations | Antigen Dilutions | | | |
|---|-------------------|--------|-----------|------|
| | 1:2 | 1:4 | 1:8 | 1:16 |
| Unfiltered cyst fluid | 1:5120 | 1:5120 | ** 1:5120 | 1:40 |
| Filtered through Seitz filter | 0 | 0 | ** 0 | 0 |
| Adsorption on Seitz filter without filtration | 0 | 0 | ** 0 | 0 |

* Experiments repeated twice with same results.

** Mean of three replications.

E. Antigenic Activity of Scolex Extracts

Saline extracts of scolices from sheep and cattle cysts when tested by the indirect hemagglutination test against standard human antiserum, in dilutions of 1:10, 1:20 and 1:40 did not show any antigenicity. Repeating this test with four different samples of scolices gave the same negative results (See Table XI).

Also, no change in the titer of the standard human antiserum was obtained when an attempt was made to adsorb antibody from 1 cc of the 1:10 dilution of the standard human antiserum by adding a drop of the scolex extract. After incubation at room temperature for half an hour this serum was re-tested against a known antigen without loss of titer.

The activity of previously antigenic hydatid cyst fluid was completely lost when scolices were lysed in it, and the supernatant used after centrifugation (Table XII).

F. Effects of Freezing, Thawing and Frozen Storage on Antigenic Content of Cyst Fluids

The effects of repeated freezing and thawing and frozen storage at -20°C on the activity of the fertile hydatid cyst fluid was investigated by freezing a sample and then thawing and testing it once every month. Samples tested in this manner for five months had not lost any of their activity. (See Table XIII).

Table XI. The Antigenic Activity of Scolex Extracts
(In Terms of Antibody Titer).

| Experiment No. | Scolex Extracts at Different Dilutions | | |
|----------------|--|------|------|
| | 1:10 | 1:20 | 1:40 |
| * I | 0 | 0 | 0 |
| * II | 0 | 0 | 0 |
| III | 0 | 0 | 0 |
| IV | 0 | 0 | 0 |

* The human O Rh⁺ erythrocytes were by a different donor.

Table XII. Antibody Titers Obtained from Scolices Lysed in the Hydatid Cyst Fluid*

| Operations | Antigen Dilutions | | |
|--|-------------------|--------|--------|
| | 1:2 | 1:4 | 1:8 |
| Hydatid cyst fluid | 1:5120 | 1:5120 | 1:5120 |
| Hydatid cyst fluid plus lysed scolices | 0 | 0 | 0 |

* Mean of three replications.

Table XIII. The Effect of Repeated Freezing and Thawing and Frozen Storage on the Antigenicity of Hydatid Cyst Fluid (In Terms of Antibody Titer).

| Date | Antigen Dilution | | | |
|-------------------|------------------|--------|----------|------|
| | 1:2 | 1:4 | 1:8 | 1:16 |
| November 27, 1963 | 1:5120 | 1:5120 | * 1:5120 | 1:80 |
| January 2, 1964 | 1:5120 | 1:5120 | * 1:5120 | 1:80 |
| February 5, 1964 | 1:5120 | 1:5120 | * 1:5120 | 1:80 |
| March 1, 1964 | 1:5120 | 1:5120 | * 1:5120 | 1:80 |
| April 6, 1964 | 1:5120 | 1:5120 | * 1:5120 | 1:80 |

* Mean of three replications.

G. Fractionation of the Antigen

Results of the indirect hemagglutination test on different lyophilized fractions of Echinococcus granulosus following the procedure of Kilejian et al (1962) are given below and in (Table XIV).

1. The freeze and thaw precipitate was negative.
2. The dialysis precipitate was active in dilutions of 1:10, 1:20, 1:40 and 1:80 at a titer of 1:2560.
3. Trichloroacetic acid precipitates gave the same titers as the dialysis precipitate, but as the yield of trichloroacetic acid precipitate (295.5 mgms) exceeded that of the dialysis precipitate (220.8 mgms), its actual antigenic activity was somewhat greater.
4. The supernatant liquid after precipitation with trichloroacetic acid and dialysis to remove the trichloroacetic acid gave a negative test.
5. The alcohol precipitate gave a negative test.
6. Supernatant liquid from the alcohol precipitate was also negative.

Results obtained from lyophilized Taenia hydatigena fractions gave the following results: (See table XV).

1. Freeze and thaw precipitate was negative.
2. The dialysis precipitate was active in dilutions of 1:10 at a titer of 1:2560 and 1:20 at a titer of 1:40.
3. The trichloroacetic acid precipitate was active in dilutions of 1:10 at a titer of 1:2560 and 1:20 at a titer of 1:40.
4. Alcohol precipitate was negative.

Table XIV. Antibody Titers Obtained with Different Fractions of Hydatid Cyst Fluid*

| Fractions tested | Antigen dilutions | | | |
|-----------------------------|-------------------|--------|--------|--------------|
| | 1:10 | 1:20 | 1:40 | 1:80 |
| Freeze and thaw precipitate | 0 | 0 | 0 | ** 0 |
| Dialysis precipitate | 1:2560 | 1:2560 | 1:2560 | ** 1:2560 |
| Trichloroacetic precipitate | 1:2560 | 1:2560 | 1:2560 | ** 1:2560 |
| Alcohol precipitate | 0 | 0 | 0 | ** 0 |

* All experiments repeated twice with same results.

** Mean of three replications

Table XV. Antibody Titers Obtained with Different Fractions of Taenia hydatigena Cyst Fluid*

| Fractions test | Antigen Dilutions | | |
|--------------------------------|----------------------|------|------|
| | 1:10 | 1:20 | 1:40 |
| Freeze and thaw precipitate | 0 ^{**} | 0 | 0 |
| Dialysis precipitate | 1:2560 ^{**} | 1:40 | 0 |
| Trichloroacetic precipitate | 1:2560 ^{**} | 1:40 | 0 |
| Alcohol precipitate | 0 ^{**} | 0 | 0 |

* All experiments repeated twice with same results

** Mean of three replications

When the above fractions of Echinococcus granulosus and Taenia hydatigena fluids were reconstituted to their original volumes and tested by the indirect hemagglutination test, all gave negative results. For this test the human O Rh⁺ erythrocytes were by a different donor.

Following a method similar to that of Hess and Deutsch (1948) hydatid cyst fluid was fractionated and the precipitate was found to be active in dilutions of 1:10. But if it was reconstituted to its original volume it gave negative results. The supernatant after removal of alcohol in a 1:2 dilution lysed the erythrocytes during sensitization, while a 1:4 dilution gave a titer of 1:2560. The original fluid was active at a dilution 1:8 at a titer of 1:2560.

H. Adsorption of Various Diluents on O Rh⁺

Erythrocytes

Because of the unexpected results obtained with scolices lysed in previously active fluid, it was decided to test the effects of addition of non-antigenic proteins to the system. To test if serum will interfere in the adsorption of the hydatid cyst fluid on O Rh⁺ human erythrocytes during sensitization of the cells, 1:2 dilutions of the hydatid cyst fluid were made using ^{human} serum of blood group AB as diluent instead of Bacto-streptolysin O buffer. A control 1:2 dilution of the hydatid cyst fluid was used simultaneously with Bacto-streptolysin O buffer as a diluent.

Another diluent was used which was 30 per cent v/v egg albumin in Bacto-streptolysin O buffer. A 1:2 dilution of the hydatid cyst fluid was made for the sensitization of O Rh⁺ human erythrocytes. Additional dilutions were prepared by adding 9 cc of the hydatid cyst and 1 cc of 30 per cent v/v egg albumin in one tube and 8 cc of the hydatid cyst fluid and 2 cc of 30 per cent v/v egg albumin in a third tube. The control was 1:2 dilution of the same hydatid cyst fluid using Bacto-streptolysin O buffer. All tests using sera of groups AB blood and egg albumin as diluents gave negative results showing that non-antigenic protein could block antigen adsorption by sensitized red blood cells.

DISCUSSION AND CONCLUSION

It appears that the host source of antigen is of no significance in the indirect hemagglutination test for hydatid disease as long as the cyst fluid is obtained from highly fertile, healthy cysts.

Concentration of the antigen does vary from fluid to fluid, however, as was found by antigen titration. This was observed with hydatid cyst fluids from various organs of sheep, cattle, burros, gerbils and man.

Hydatid cyst fluids from camels and burrow gave inconsistent titers against standard antiserum. Some cyst fluids gave a low titer, while others gave the same titer as other hydatid cyst fluids. Although these fluids were all from cysts containing at least some scolices, perhaps this difference could be explained in that camels and burros are usually slaughtered at older ages, and thus the cysts may not be as healthy as those of other hosts.

Infertile hydatid cyst fluids from natural infection of cattle and secondary infection of mice were also examined with interesting results. Large infertile cysts in cattle represent cysts which in all probability were originally fertile but in which the scolices had subsequently degenerated, resulting in an infertile cyst. Fluids from such cysts were consistently non-antigenic or gave very low serum titers. It was not known from these results, however, whether or not scolices alone were involved in the production of antigen and its release into the fluid. The possibility remained that the germinal membrane could also be involved in the production of antigen even before the scolices

developed within cysts. Schwabe et al (1964) showed that scolices seldom formed in secondary cysts of rodents which were less than 1 cm. in diameter. Infertile hydatid cyst fluid from secondary infection of mice where the scolices had not yet formed was used therefore, as a source of antigen. In four experiments, pooled cyst fluids from two rodents were found to contain antigenically active fluid. These two positive results could be explained if certain cells of the germinal membrane had become differentiated to antigen producing cells. Perhaps differentiation had not yet taken place in the case of the other two pools which were antigenically negative. Further investigation on this subject is needed to know the exact situation.

Bacigalupo (1925) did not inactivate the antigen involved in the complement fixation test and the Casoni intradermal test for hydatid disease by heating the hydatid cyst fluid for five minutes at 100°C. In contrast to his findings, heating the fluid at 80°C for five minutes reduced its activity in the indirect hemagglutination test. Heating it at 100°C for five minutes completely inactivated the fluid. This would seem to indicate that the antigen involved in the indirect hemagglutination test is chemically different from that in the complement fixation and Casoni intradermal test.

Dennis (1937) was able to obtain positive Casoni intradermal tests, precipitin tests, and complement fixation tests using a trichloroacetic acid precipitate of hydatid cyst fluid after sterilizing it, by filtering through a Seitz filter. Garabedian et al (1957a) noted, however, that filtering hydatid cyst fluid through asbestos Seitz filters

gave entirely unsatisfactory antigen in the indirect hemagglutination tests. Pellegrino and Rodrigues (1960) in testing the influence of Seitz filtration on the activity of Schistosoma mansoni adult worm antigen found it produced smaller wheals in the intradermal test after filtration. The nitrogen content was drastically reduced as a result of removing most protein nitrogen. In this work it was found that the antigen was completely lacking in the cyst fluid filtrate after Seitz filtration. In an attempt to elucidate these results, antigen was subsequently exposed to Seitz filter pads without filtration. This procedure also caused a complete loss of antigenic activity indicating adsorption rather than filtration loss. These results suggested further that a chemical difference exists between antigen for the intradermal and complement fixation tests on the one hand and the indirect hemagglutination test on the other.

In further experiments it was found that repeated freezing and thawing and frozen storage did not affect the antigenicity of the hydatid cyst fluid for at least five months. The antigen gave uniform results by the indirect hemagglutination test each month it was tested.

Kagan et al (1960b) extracted the scolices obtained from pig cysts with saline. This extract was used by them as an antigen in the indirect hemagglutination test in testing sera of several different patients infection with hydatid disease. Positive results were obtained, although the titers were not as high as those obtained with cyst fluid antigen. Following their method of extraction exactly, except that cattle and sheep scolices were used instead of pig scolices, negative

results were obtained by the indirect hemagglutination test. Extracting four different samples of scolices showed no antigenic activity even when concentrated extracts of the scolices were used. This difference in results cannot be accounted for except possibly in the fact that Kagan used some sera with higher titers than the standard antiserum used in this study. In further experiments, antibody could not be adsorbed from the standard antiserum by scolex extracts suggesting further that scolex extracts were lacking in antigenic activity.

In a third attempt to demonstrate antigen in scolices, scolices were lysed within highly antigenic cyst fluid in an effort to enhance its antigen titer. Surprisingly, lysing the scolices in active hydatid cyst fluid destroyed antigenic activity already possessed by the fluid alone. One would have predicted that the activity of the fluid would either have remained the same or have been enhanced by substances released by the scolices. However, since the fluid lost its activity, the possibility had to be considered that the scolices had released large quantities of non-specific substances which, however, were competitively adsorbed with the antigen on the erythrocytes during sensitization. This could in effect block the surface of the erythrocytes for the adsorption of the antigen or the lysed scolices could have released substances that precipitated the antigen from the fluid, or the released substances could have interfered in some other way in the agglutination of the erythrocytes.

To test these various theories, two different foreign proteins non-antigenic in the hydatid system were used as diluents for the hydatid cyst fluid antigen in place of the Bacto-streptolysin O buffer.

Egg albumin and normal human serum both interfered in the indirect hemagglutination test and destroyed the antigenicity of previously antigenic fluids. This would seem to indicate that these proteins were adsorbed on the human erythrocytes thus blocking the surface of the cells for the antigen adsorption. This same mechanism could account for the results obtained with the lysed scolices.

In attempts to free hydatid cyst fluids of non-specific reactive substances, it was shown that during dialysis of hydatid cyst fluid the activity of the fluid was retained. This proved that inorganic ions, and small molecules were not required for the activity of the fluid. A precipitate which formed during dialysis and which behaved as a protein on electrophoresis, was active by the indirect hemagglutination test. But it was not as active as the fluid, because when it was reconstituted to its original volume activity could not be demonstrated.

The trichloroacetic acid precipitate prepared following the method of Kilejian et al (1962) was active by the indirect hemagglutination test. It was more active than the dialysis precipitate, but less active than the whole hydatid cyst fluid. Trichloroacetic acid is a protein precipitant, and since proteins are delicate they may have been denatured partially during the fractionation process. Some evidence for this has been obtained by Schwabe and Koussa (unpublished data). Partial denaturation could account for the loss of activity observed. The infrared spectrum obtained by Kilejian et al (1962) with the trichloroacetic acid precipitate indicated its protein nature.

The alcohol precipitate from the supernatant liquid of the trichloroacetic acid precipitate has been shown to consist of a mucopolysaccharide by Kilejian et al (1962). This precipitate gave negative results in the indirect hemagglutination test indicating that the antigen is not a mucopolysaccharide. This is supported by the fact that Taenia hydatigena cyst fluid which has been shown by Schwabe and Koussa (unpublished work) to be free from hydatid mucopolysaccharide, was equally useful as an antigen in the indirect hemagglutination test for hydatid disease. These observations indicate that the active antigen in this test is protein and not mucopolysaccharide.

One of the most interesting findings in this study was that the indirect hemagglutinating antigen is group specific in that Taenia hydatigena fluids substituted for hydatid cyst fluids in the test. Schwabe (unpublished data) had previously shown that Taenia hydatigena fluid also reacted with hydatid antisera on agar gel diffusion. Similar lyophilized fractions of Taenia hydatigena were tested by the indirect hemagglutination test. Both the dialysis precipitate and the trichloroacetic acid precipitate were active. This activity, however, was less than that obtained from analogous fractions of Echinococcus granulosus. This reduced activity could conceivably be due to the fact that the Taenia hydatigena fractions were stored for one year in the lyophilized state before use, whereas Echinococcus granulosus fractions were freshly prepared.

Using the alcohol fractionation method described by Hess and Deutsch (1948) for protein in bovine sera, a protein fraction of hydatid cyst fluid was obtained which was active in the indirect hemagglutination test. But this activity was less than that of the original fluid, and the supernatant liquid also possessed some activity. This indicated incomplete precipitation of the antigen or partial denaturation. Possibly a higher concentration of alcohol could result in a more complete precipitation of the antigen, but this also may lead to denaturation. This is a subject for further study.

From the above discussion it would seem that the active antigen in the indirect hemagglutination test is protein in nature and that it is shared by at least closely related tapeworms. It is fairly stable as evidenced by its resistance to heat, storage, freezing and thawing and various chemical protein precipitants. It differs in several properties from the antigen responsible for activity in the complement fixation test, Casoni intradermal test and precipitin test. The results obtained by previous workers suggest that the antigen in these latter tests may be the mucopolysaccharide of hydatid cyst fluid and tissues.

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