The chemical and immunological properties of Sarcoma I tumor specific antigen
by Marilyn Jo Zanger Aden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
Montana State University

Abstract:
The tumor specific antigen of Sarcoma I ascites tumor cells was solubilized by extraction with the nonionic detergent, Triton X-100. This was found to be a relatively efficient method of extraction, since large yields of antigenic material could be obtained. This preparation was primarily lipoprotein in nature having 850ug/ml of protein, 52ug/ml of carbohydrate and no hexos- , amine. The percentage of lipid present, as determined, was 42.5 per cent. The Sarcoma I-Triton solubilized lipoprotein (SaI-TSL) material was found to be heterogenous by ultracentrifugal and electrophoretic criteria. A single, broad peak was observed upon sedimentation velocity analysis. Disc gel electrophoresis revealed the presence of more than 6 distinct bands. The average sedimentation coefficient estimated for this material was S20,w= 2.94, suggesting a relatively low molecular weight substance. Attempts to produce low-zone tolerance in both adult and neonatal A/Jax mice with the SaI-TSL preparation failed. In fact, immunization occurred with all time-dose combinations employed.

The greatest percentage of survivals was observed utilizing small, multiple injections in neonatal mice. Possible reasons for failure to produce low-zone tolerance are: utilization of an aggregated antigen, the selection of inappropriate time-dose schedules, and/or the inappropriateness of the assay used to detect tolerance production.

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

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March, 1972

ACKNOWLEDGMENT

I would like to express my appreciation to Dr. John W. Jutila for the guidance, patience, and encouragement he gave throughout this investigation. My thanks to committee members, Drs. Frank S. Newman and William D. Hill, for their suggestions and assistance in the preparation of this manuscript. I would also like to thank my husband, David, for his encouragement, patience, and helpful suggestions during the course of this study. I am also grateful for the research and stipend support received from training grant AI 131-09.

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ABSTRACT

The tumor specific antigen of Sarcoma I ascites tumor cells was solubilized by extraction with the nonionic detergent, Triton X-100. This was found to be a relatively efficient method of extraction, since large yields of antigenic material could be obtained. This preparation was primarily lipoprotein in nature having 850ug/ml of protein, 52ug/ml of carbohydrate and no hexosamine. The percentage of lipid present, as determined, was 42.5 per cent. The Sarcoma I-Triton solubilized lipoprotein (SaI-TSL) material was found to be heterogenous by ultracentrifugal and electrophoretic criteria. A single, broad peak was observed upon sedimentation velocity analysis. Disc gel electrophoresis revealed the presence of more than 6 distinct bands. The average sedimentation coefficient estimated for this material was $S_{20,w}$= 2.94, suggesting a relatively low molecular weight substance. Attempts to produce low-zone tolerance in both adult and neonatal A/Jax mice with the SaI-TSL preparation failed. In fact, immunization occurred with all time-dose combinations employed. The greatest percentage of survivals was observed utilizing small, multiple injections in neonatal mice. Possible reasons for failure to produce low-zone tolerance are: utilization of an aggregated antigen, the selection of inappropriate time-dose schedules, and/or the inappropriateness of the assay used to detect tolerance production.

INTRODUCTION

General Considerations

Histocompatibility (H) antigens are substances associated with the plasma membrane of cells of some, but not all, members of a species. This is substantiated by observations made with flourescent alloantibody (27,85), agglutinating alloantibody (1,28), antibody absorptions before and after cell rupture (40), and purified fractions (97). These histocompatibility antigens are responsible for transplantation immunity, i.e., they will induce an immune response when introduced into another member of the same species. Thus, they are commonly termed transplantation antigens and seem to be present on all cells (77) and develop early in ontogeny (6,107).

Histocompatibility antigens (H antigens) are the products of histocompatibility genes and in each species which has been carefully studied, there is a single strong histocompatibility locus controlling the rapid rejection of allografts. These are represented by the H-2 locus in mice (of the more than 15 other loci present), the HL-A locus of man, the Ag-B (H-1) locus of rats, and the B locus of chickens.

The modern era of tumor immunology began approximately 10 to 15 years ago with the demonstration that chemically-induced mouse sarcomas possess antigens which are lacking in normal cells of the host strain of tumor origin (98,63). Numerous investigations have since

been performed on many murine tumors. These tumors provide an abundant source of transplantation antigens, which makes it a useful model for study in human systems. Chemically-induced tumor antigens are designated tumor-specific transplantation antigens (TSTA) because of their capacity to elicit rejection responses against transplanted tumor cells in syngeneic hosts (63,99). Chemically-induced tumors were found to possess TSTA which are unique for each neoplasm (63), whereas tumors which are viral-induced differ, in that all tumors induced by the same virus possess the same tumor specific antigens (96,108). The relationship between TSTA and normal H-2 antigens is of interest because of the possible role that these surface components may play in cell recognition. Recent studies by Haywood and McKhann (41) on murine sarcoma cells and by Ting and Herberman (112) on polyoma virus-induced tumors indicate an inverse relationship between TSTA and normal histocompatibility antigens, i.e., highly immunogenic tumors had quantitatively less H-2 antigen on their surfaces.

The immunological consequences of the introduction of cells bearing histocompatibility antigens into allogeneic recipients can be varied. A hastened rejection of allografts or tumors (the second set phenomenon) may occur. In vitro techniques (42) detecting immunity to tumor antigens have shown this response to be primarily of a cellular nature, involving immune lymphocytes which can kill neoplastic

cells (62).

Among the available methods for inducing tumor specific transplantation immunity are (i) subthreshold doses of living tumor cells (23), (ii) irradiated tumor cells (23,47), (iii) living tumor cells intradermally (a site promoting growth and regression) (23,65,66,114), (iv) living tumor cells followed by amputation of the growing tumor (45,88), (v) tumor cells mixed with Freund's adjuvant (7), (vi) tumor cells mixed with Mycobacterium bovis intradermally (118), (vii) the coupling of tumor cells to highly antigenic foreign protein (13,14) or chemicals (15), (viii) tumor cells grown in tissue culture (23), and (ix) cell free extracts of tumor cells (see immunological properties of tumor antigens).

Another consequence of the introduction of living cells or extracts of cells is related to the protective effect of circulating antibodies on tumor cells from an allogeneic donor (61). These antibodies are often cytotoxic when tested alone against tumor cells in the presence of complement, but under certain in vivo conditions protect neoplastic cells from destruction (54). This process is known as immunological enhancement. A number of recent articles and reviews (2,8,55) relating to this subject exist.

Yet another consequence of the introduction of H-antigens into hosts is immunological tolerance. Following the injection of large

doses of soluble or particulate transplantation antigen, a state of specific unresponsiveness develops in the treated animal. Living cells bearing tumor antigens are most effective for developing tolerance since they proliferate and increase as well as maintain a high level of tolerizing antigen. In 1962, Dresser (21) reported that injection of mice with small amounts of the supernatant from an ultracentrifuged solution of bovine gamma globulin (BGG) delayed the clearance of a subsequent larger dose of BGG. He believed that this delayed antigenic clearance represented a form of immunological paralysis or tolerance. In 1963, Medawar (78) delayed the rejection of allogeneic skin grafts by pre-injecting hosts with a semi-soluble antigen preparation. He noted that the semi-soluble preparation prolonged graft survival more effectively than the particulate preparation and theorized that a potent soluble antigen might be capable of provoking a high degree of tolerance. According to Mitchison and Dresser (22,81) various protein antigens used at relatively low doses in animals which are immunologically immature, or in mature animals treated with immunosuppressive agents, can produce a paralysis of the immune response. The low dose of antigen utilized is thought to be sufficient to initiate the tolerogenic system, but inadequate to result in processed products. As summarized by Leskowitz (67), a soluble antigen is thought to be tolerogenic since it is not easily

phagocytized and hence, it is not processed by the macrophages. In contrast, high dose tolerance, in which extremely large doses of antigen are utilized, is thought to overwhelm the phagocytic capacity of the macrophages, permitting preferential activation of the tolerogenic system.

Properties of Histocompatibility Antigens

Progress in determining the chemical nature of transplantation antigens has been very slow. Many laboratories are presently engaged in the investigation of the chemistry of membranes from a variety of cells and subcellular organelles. In these studies, the nature of the membrane components presents difficulties in solubility. Also, the fact that the techniques presently available to solubilize these substances yield only modest amounts of antigenic material as a minor component in an exceedingly large heterogenous array of contaminating substances is a definite obstacle.

Attempts to solubilize antigens from their membrane-associated site have markedly increased from the time of the discovery by Medawar (78) that nonparticulate transplantation antigens induce prolonged graft survival and the observation that these antigens can be easily fractionated and analyzed only in the purified state (51).

Varying definitions for solubilized transplantation antigens exist. Some authors consider their antigen solubilized if it does

not precipitate upon being centrifuged at 100,000 X g for 1 hour. However, recent work by Rapaport (100) contradicts this criteria by showing that antigen which is not precipitable during centrifugation at 100,000 X g, is at 200,000 X g. Ultrastructural analysis of the 200,000 X g material consisted of membrane fragments. Others (15) distinguish solubilized from "stabilized" materials on the basis of the supposed chemical complexity of the latter, the requirement that the solubilized material need no additives present to retain its solubility in aqueous solution, and the sedimentation of "stabilized" preparations in the absence of the solubilizing agent. At any rate, a soluble antigen should be defined as one that exists in true solution in aqueous solvents.

To solubilize and purify H-antigens, which comprise less than 1 per cent of the cell membrane, many different procedures have been used. Some investigators simply utilize homogenization coupled with high speed centrifugation to obtain biologically active preparations of crudely solubilized material (94,7). Detergents such as Triton, deoxycholate, and sodium dodecylsulfate, by itself, or in the presence of starch stearate have been employed by a number of workers (10,18, 19,37,57,58,60,79).

In 1960, Kandutsch (57) found that treatment of particulate fractions of Sarcoma I mouse ascites cells with deoxycholate or with

5 per cent Triton X-100 yielded an antigenic material, whereas treatment with sodium dodecylsulfate yielded inactive preparations. Triton X-100 has also been used successfully in solubilizing erythrocyte membranes and has been found to yield a product of much higher activity than other detergents structurally related to X-100 (80). Kandutsch and Stimpfling (58) described a water-soluble isoantigenic lipoprotein which had been extracted with Triton X-100 from Sarcoma I tumor cells and then solubilized by the action of snake venom, presumed to be rich in the enzyme phospholipase A. Graff and Kandutsch (29) later demonstrated that this water soluble lipoprotein was highly antigenic. Recently, Kandutsch et al. (60) extracted the membrane fraction of mouse Sarcoma I with 0.25 per cent cholate in the presence of 3M potassium chloride and obtained an insoluble antigen, which was then solubilized with Triton X-114.

Although several investigators have failed in attempting solubilization with deoxycholate, Metzgar et al. (79) and Bruning et al. (10) were successful in extracting antigenic determinants of the human HL-A system and Manson and Palm (74) from mouse microsomal lipoproteins. Davies et al. (18,19) and Hammerling et al. (37) have solubilized both H-2 and HL-A antigens using a combination of sodium dodecylsulfate and starch stearate.

Another group of extraction reagents which have been employed are the organic solvents. Several investigators have utilized butanol for this purpose (57,74,89). Harris et al. (38) combined Triton X-100 extraction with butanol treatment to prepare a complex soluble antigen from rabbit lymph node and spleen cells. Other workers (33) have used solvent extraction with ether:benzene (2:1, V/V). In addition, sheep red blood cell membranes have been extracted with a number of organic solvents (9,26).

Transplantation antigens may also be obtained in a water soluble state by exposure to low frequency sound. The activity of the liberated antigens depends upon the conditions of sonication. Potent antigens are released utilizing low-frequency, low-intensity sound, whereas only small amounts of less active antigens are liberated using high-frequency sources. Thus, Billingham (5) and Haughton (39) did not obtain very active antigenic material utilizing high-intensity sound. However, brief exposure to low-intensity sound liberated histocompatibility antigens from a variety of murine, guinea pig, rat, canine, and human tissues (51). Kahan et al. (48-52) and Holmes et al. (44,45) obtained antigenically active extracts from neoplastic and normal guinea pig tissues utilizing this technique as did Koene et al. (64) with mouse H-2 antigens and Kahan et al. (53) again with HL-A antigens.

Autolytic digestion has also been used for H-antigen solubilization, either alone, or followed by proteolysis (17,25,33,34,91, 103). Colombani et al. (11) found that the addition of ficin or papain yielded little or no additional active soluble antigens to autolyzed HL-A antigens. However, other investigators have greatly enhanced the activity of their preparations using this combination (70,92).

Enzymatic methods of degradation of crude membrane-derived material have for some time been the most successful in solubilization. A number of recent articles exist describing the solubilization of H-2 and other alloantigens utilizing enzymatic digestion with papain (11,12,16,17,25,31,32,35,36,71-73,82-84,90,93,103-105,109-111,113,116,117). Halle-Pannenko et al. (35) obtained the best results with papain, but also had reasonably high antigenic yields with trypsin. Ficin preparations, however, had only very weak antigenic activity.

The most promising method developed thus far appears to be the potassium chloride (KCl) technique described by Reisfeld et al. (102). Combining this procedure with preparative, discontinuous polyacrylamide electrophoresis, they obtained higher recoveries of potent HL-A antigens from lymphoid cell lines than with any other method. Etheredge and Najarian (25) also used this method successfully with

HL-A antigens from spleen cells. Mann and Fahey (73), however, obtained poor yields of HL-A antigens from lymphoid cells utilizing KCl or EDTA.

Chemical and Physical Properties of Tumor Antigens

In 1961, Herzenberg and Herzenberg (43), isolated much of the H-2 antigenic activity of mouse liver in a lipoprotein fraction from nuclear and cellular membranes. They found their material to contain less than 1 per cent carbohydrate and detected no hexosamine. These values were lower than those reported by Kandutsch and Reinert-Wenck (56) for their preparations of Sarcoma I antigen. Kandutsch and Stimpfling (58) also found their preparation of Sarcoma I to consist of lipoprotein upon solubilization of the antigenic material in Triton. They found the Triton-treated material to be essentially homogenous by electrophoretic and ultracentrifugal criteria. A sedimentation coefficient in the presence of Triton (S_{20}= 2.65) suggested a low molecular weight. They also detected only a small amount of carbohydrate.

Solubilization of HL-A specificities with starch stearate and sodium dodecylsulfate by Davies et al. (18), resulted in a product of molecular weight in excess of 200,000. However, when these antigens were solubilized by autolysis and papain digestion, molecular weights in the region of 50,000 were found (17). Utilizing the smaller molecular weight preparations, these investigators were able to

construct a column separation profile for the various H-2 antigens (16) and have begun a similar profile for the HL-A antigens (11). Recently these authors have published articles comparing these two molecular weight forms and have presented evidence that some H-2 specificities reside on the same molecule (19,37).

Histocompatibility antigens released by sonication have no carbohydrate moiety, at least not detectable by the method analyzed (25). Kahan et al. (53) have found that the antigenic activity of various H-substances solubilized by sonication is a relatively homogenous fraction on acrylamide gel electrophoresis. These purified components have characteristic and reproducible amino acid sequences for each line and lack any detectable carbohydrate or lipid moieties. The active portion of guinea pig transplantation antigens solubilized by sonication was found to be electrophoretically inhomogenous on polyacrylamide gel due to the presence of contaminant substances with similar electrophoretic mobilities (52). Koene et al. (64), utilizing the same technique for solubilization of mouse H-2 antigens, detected a single band on polyacrylamide gel electrophoresis. However, utilizing starch-block electrophoresis, two bands were detected. Only one was found to be antigenically active and had a molecular weight of 60,000.

Crude extracts of KCl-treated HL-A antigens have been found to have a relatively low content of lipids and lipoprotein. These materials can be directly electrophoresed on acrylamide gels, thus allowing for more rapid purification. Reisfeld et al. (102) isolated the antigenic activity of HL-A antigens in only one of several fractions eluted from preparative acrylamide gel using this technique. Upon re-electrophoresis on analytical acrylamide gel, the active fraction contained only one electrophoretic component, as had been previously shown with HL-A antigens solubilized with sonication (101). Etheredge and Najarian (25), however, obtained very low yields of HL-A alloantigens using this technique. Varying molecular weight substances (45,000 and 14,000) were released with KCl. Mann and Fahey (73) also obtained very low yields and low levels of activity of HL-A antigens with KCl and EDTA treatments. Alloantigenic activity was detected only in fractions having a molecular weight greater than 200,000. Materials solubilized with Tris-2-hydroxy-3,5-diiodobenzoate (TIS) provided intermediate yields of antigen and chromatographed on Sephadex and electrophoresed on polyacrylamide gels as a single component with a molecular weight of approximately 160,000. Sodium dodecylsulfate, however, was capable of separating this material into two smaller components with molecular weights of 60,000 and 90,000. Papain-solubilized HL-A alloantigens were found to have a molecular weight of approximately 60,000 and these investigators found that the

best yields were provided by this method (73). KCl and EDTA-solubilized HL-A antigens, but not TIS-solubilized materials, were shown to have similar electrophoretic patterns to those which had been papain treated.

Strober et al. (109,110) have also solubilized mouse H-2 transplantation antigens with papain and have found that the alloantigenic specificities controlled by the H-2 locus were confined to a single peak after chromatographic fractionation. Etheredge and Najarian (25) have found that papain proteolysis of human HL-A substances released a material of approximately 48,000 molecular weight. Autolysis produced HL-A substances of varying molecular weights ranging from greater than 800,000 to below 50,000. Miyakawa and workers (82,83, 111) have recovered high yields of 48,000 molecular weight protein substances from papain solubilized HL-A alloantigens, which were also relatively homogenous by electrophoretic criteria. These workers recovered 30 to 37 per cent of the HL-A antigenic activity for each isolated specificity studied utilizing this method.

Halle-Pannenko et al. (35) obtained 49 per cent of the original activity in a papain-solubilized preparation of mouse tumor cells. They found this fraction to be largely lipoprotein and upon acrylamide gel electrophoresis, a diffuse active band was observed. A number of studies utilizing papain for solubilization of H-antigens

have indicated that many of these substances are glycoproteins with a molecular weight of about 50,000.

Nathenson and workers (12,31,32,90,93,106,116) in a number of recent articles have chemically characterized H-2, HL-A, and tumor specific antigens utilizing enzymatic digestion of cell membranes with papain. In studies on mice, two fragments were isolated. These purified fragments were homogenous by disk gel electrophoresis. Class I fragments were glycoproteins of approximately 57,000 molecular weight with 80 to 90 per cent protein, 4 per cent neutral carbohydrate, 3 to 4 per cent glucosamine, and 1 per cent sialic acid. Class II fragments were glycoproteins of approximately 35,000 molecular weight and were of similar chemical analysis to the Class I fragments. No phosphate or lipid was present in either preparation. In comparing the carbohydrate composition of the glycoprotein fragments from two murine strains allelic at the H-2 locus, no differences could be found. Peptide maps, however, showed small but reproducible differences, suggesting that protein plays a central role in determining the expression of H-2 alloantigenic specificity. Comparative studies between papain-solubilized human HL-A alloantigens and mouse H-2 antigens showed similarity in solubilization, chromatographic and electrophoretic properties. Comparing the amino acid content of the alloantigens of these two species added further evidence for

similarity at the molecular level. Mouse alloantigenic preparations from normal and tumor cell sources, both of which carry the same H-2 specificities, were found to have not only similar serological profiles by several criteria, but also similar amino acid and chemical analyses, molecular weight, chromatographic, and electrophoretic properties (116). These authors have also developed a method for determining the distribution of H-2 alloantigenic specificities on the papain solubilized fragments. Using a similar method, Cullen and Nathenson (12) provide evidence that several H-2 alloantigenic specificities reside on the same fragment.

Immunological Properties of Tumor Antigens

Particulate or crudely soluble H-antigens and tumor specific antigens (TSA), as well as soluble antigens, are effective in producing a number of immune reactions in animals.

Monaco et al. (86), utilizing cell-free H-2 antigens prepared by homogenization and centrifugation were able to cause prolonged graft survival in allogeneic mice with repeated injections of the sensitizing antigen. They found that very small amounts of this antigen were capable of causing significant sensitization and that the extracted antigen was as potent as the intact cells from which it was prepared. McCollester (76) has recently described a method for isolating the surface membranes of Meth A tumor cells. He found that

this tumor ghost preparation could produce immunity in BALB/c mice against a challenge of Meth A tumor cells. Oettgen and workers (94,7) have obtained crudely solubilized guinea pig tumor antigens by homogenization and centrifugation. They have reported delayed hypersensitivity reactions, inhibition of macrophage migration, and transplantation immunity to these "soluble" antigens.

Utilizing soluble tumor specific antigens obtained by treatment of Sarcoma I with Triton X-100 and phospholipase A, Kandutsch and Stimpfling (58) were able to show that these antigens were active in hemagglutination inhibition, hemagglutination production, and enhancement tests. However, preliminary skin graft tests for the production of cellular immunity indicated weak or no activity. Graff and Kandutsch (30) later tested the ability of these identically prepared tumor antigens to induce low-zone tolerance to skin grafts. Under the conditions of the experiments, tolerance was not produced, but, rather some time-dose combinations caused accelerated graft rejection.

Holmes and workers (44,45,52) demonstrated that guinea pigs produced delayed-type hypersensitivity reactions upon intradermal challenge and increased resistance to specific tumor challenge following the injection of soluble tumor antigen. Mouse histocompatibility antigens have also been solubilized by sonication and studied for biologic activity by Koene et al. (64). These investigators

found that crudely soluble, partially purified antigen given in large doses was able to induce antibody formation and prolong graft survival (active enhancement). If given in the mid-dose range, an accelerated graft rejection occurred. More purified fractions were also found to be immunogenic.

Halle-Pannenko et al. (34;36), using autolytic digestion and digestion with papain to solubilize mouse tumor cells observed a prolongation of survival of allogeneic skin grafts after immunization of recipients. If cyclophosphamide was also administered with low doses of these antigens, a synergistic effect was noted.

The immunogenic properties of papain-solubilized alloantigenic preparations from mouse spleen cells were studied by Graff et al. (31,32). In the first of a series of studies, an ammonium sulfate precipitation fraction was found to be capable of inducing allograft immunity. Intravenous injections of single and multiple doses of the preparation were not capable of producing tolerance. In a later study, histocompatibility antigens at various stages of purification were tested for their ability to cause accelerated rejection of subsequent skin grafts. All fractions tested were shown to possess H-2 immunogenicity. Strober et al. (109,110) also employed papain to digest mouse transplantation antigens. Only one of three peaks (F_2) eluted from a Sephadex G-150 column was capable of producing signifi-

cant acceleration of skin graft rejection in co-isogenic strains that differ at the H-2 locus. However, all three peaks (F_1, F_2, and F_3) produced significant acceleration of skin graft rejection in non-co-isogenic strains that differ at H-2 and non-H-2 loci. The association of alloantigenic specificities and transplantation antigens controlled by the H-2 locus in peak F_2 suggested that both are present on the same molecules. This is in agreement with recent studies by Cullen and Nathenson (12).

Introduction to Thesis Problem

Solubilization of histocompatibility substances has been accomplished utilizing a number of techniques. Biochemical and biological studies of these solubilized materials have produced a large number of varying results. The nonspecificity of these solubilization techniques suggests that differences in products may be attributed to the differences in techniques. Solubilization of these substances is necessary for a number of reasons. It is only in the soluble form that H-substances can be easily purified and characterized. The purification and chemical characterization of these antigens could lead to the development of potent tumor-specific immunogens useful clinically in tumor prophylaxis and immunotherapy. Preliminary studies (47) have shown that irradiated Sarcoma I tumor cells are immunogenic in A/Jax mice, hence they must bear a tumor specific transplantation

antigen. Moreover, immunity to SaI tumor challenge can be produced with a streptomycin sulfate precipitated extract of tumor cells (46).

It is the purpose of this investigation to extend these findings to include the chemical and immunological properties of Sarcoma I TSTA solubilized by a combination of streptomycin precipitation and Triton X-100 treatment.

MATERIALS AND METHODS

Mice

Inbred conventionally reared A/Jax mice were used throughout this study. These mice were originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and have been maintained on sterilized Purina 5010C and acidified water *ad libitum* with occasional feeding of Quaker Rolled Oats.

Tumor

An ascites form of Sarcoma I (SaI), indigenous to strain A mice, was maintained in serial passage in A/Jax mice by intraperitoneal injections. This tumor was originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. SaI is a tumor that originated in 1947 in a mouse of the Strong A strain that had been treated with dibenzanthracene (69). The tumor grows progressively in 100 per cent of the A/Jax subline mouse following challenge with doses as small as 100 cells. For a detailed study of the behavior of SaI ascites tumor in the A/Jax mouse, see the work by Baker *et al.* (4).

Isolation of Immunizing Lipoprotein (ILP) from Sarcoma I

SaI ascites tumor was aseptically extracted from groups of 10 to 20 A/Jax mice which had been injected 10 to 14 days previously with SaI cells (see SaI tumor challenge in materials and methods for

details concerning tumor passage). The mice ranged in age from $1\frac{1}{2}$ to 4 months, with each group of mice used being of approximately the same age and weight. The mice were sacrificed by cervical dislocation and an incision was made along the ventral aspects of the mouse, exposing the facia. Entrance into the area underlying the facia was gained utilizing a 10 ml syringe equipped with a 22-guage needle with which tumor cells were aseptically removed and placed into cold sterile tubes. Eight or 10 ml of cold phosphate buffered saline (PBS), pH 7.2, to which 250 USP units of heparin (Wolins Incorporated, Farmingdale, New Jersey) was added, was injected, and the mouse was shaken by the tail several times to suspend the SaI cells lodged in the corners of the peritoneal cavity. This PBS-cell mixture was removed in the same manner as the tumor cells, added to the original volume, and centrifuged in the cold at 3500 rpm for 15 minutes in a Sorvall RC2-B centrifuge. The supernatant fluid was drawn off and discarded. To prepare an immunizing lipoprotein from Sarcoma I, the packed cells were washed with 4 volumes of cold distilled water and centrifuged in the cold at 3500 rpm for 15 minutes (see Figure 1). The sedimented cells from this step were resuspended in 12.5 volumes of cold distilled water, extracted at 4C for 2 hours, and then centrifuged at 8000 X g for 20 minutes. The supernatant was precipitated with 1000ug/ml dihydrostreptomycin sulfate (Nutritional Biochemical

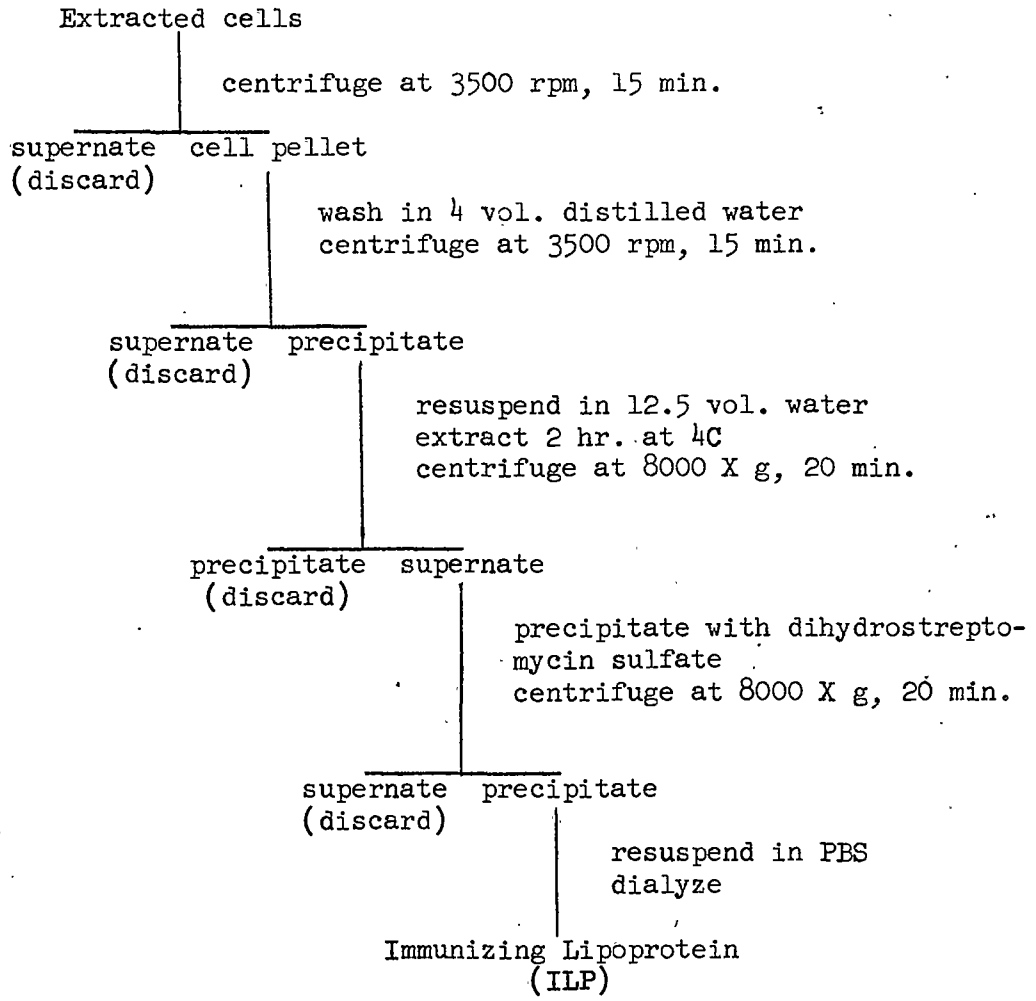

Figure 1. Flow diagram of preparation of Immunizing Lipoprotein (ILP). (See materials and methods for details).

Corporation, Cleveland, Ohio) and centrifuged at 8000 rpm for 15 minutes. The pellet was then resuspended in PBS (4.6 times the original packed cell volume (PCV) and dialyzed overnight against 2 changes of PBS. This preparation was then pipetted into 10 ml aliquots and frozen until use for immunization purposes. For use, 1 or more aliquots were thawed at room temperature and the material in each aliquot pipetted until a uniform suspension was obtained.

Isolation of Triton Soluble Lipoprotein (TSL) from Sarcoma I

Solubilization was performed on the ILP preparation isolated as described above, with the exception that the final pellet was resuspended in a smaller volume of PBS (2 to 3 times the original PCV). In addition, the ILP material was not frozen, but used directly after the dialysis treatment.

The procedure adopted for use in solubilizing the ILP material was essentially the same as the technique employed by Ogburn et al. (95) (see Figure 2). The ILP preparation was centrifuged in the cold for 1 hour at 100,000 X g in a Beckman Model L-2 preparative ultracentrifuge with the type 50 Titanium head. Throughout the rest of the solubilization procedure, aseptic techniques and sterilized equipment were used whenever feasible. In a typical preparation, 4 ml packed cell volume of the cell pellet was suspended in 100 ml of distilled water. A few crystals of thymol and 8 ml of Triton X-100

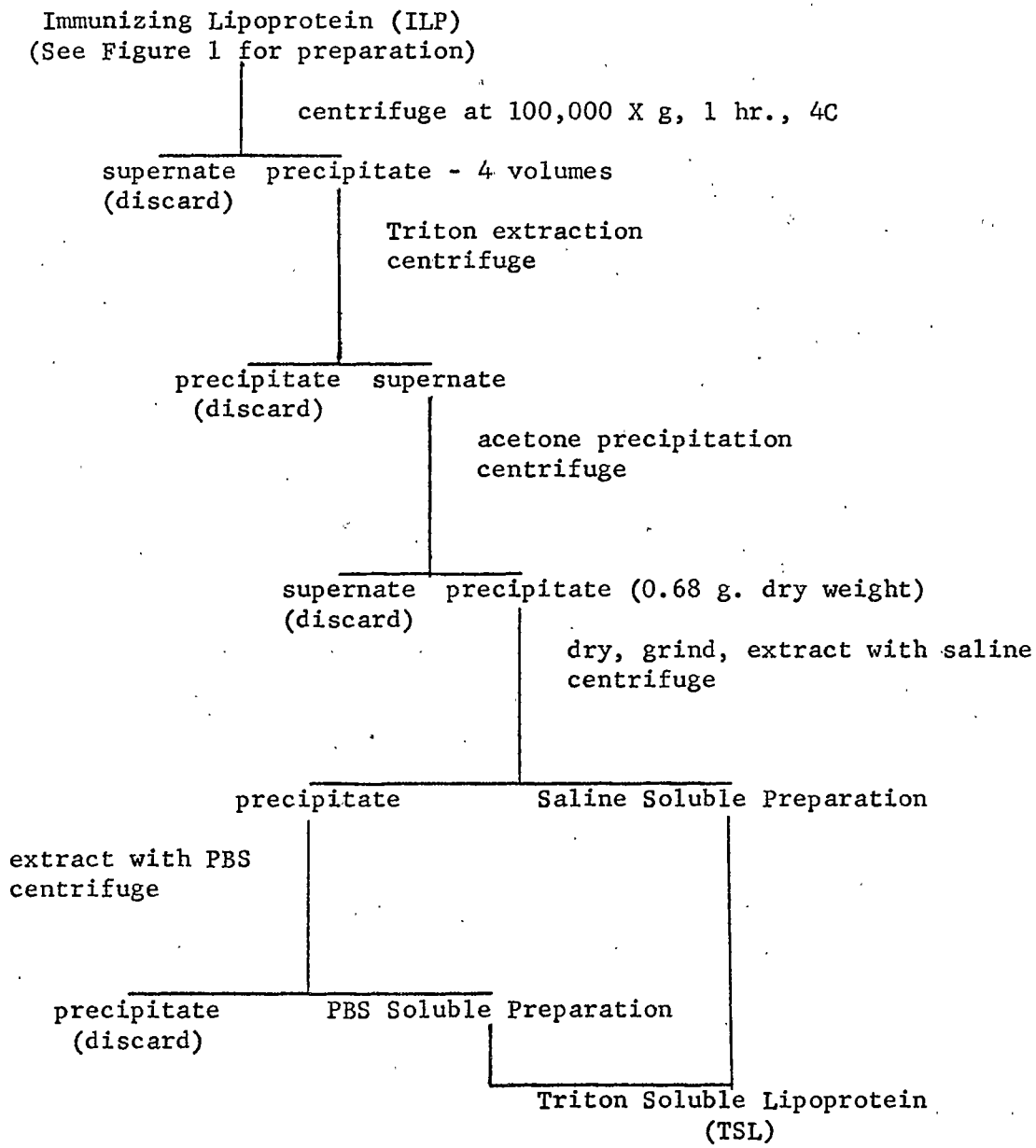

Figure 2. Flow diagram of preparation of Triton-Soluble Lipoprotein (TSL). (See materials and methods for details).

(Rohm and Haas, Philadelphia, Pennsylvania) dissolved in 52 ml of distilled water were added. The pH of the suspension was adjusted to 7.5 with tris-HCl buffer, pH 8.6 (solution A--0.2M Tris, Nutritional Biochemicals Corporation, Cleveland, Ohio; solution B--1.0M HCl; solution C--12.2 ml of solution B was added to 250 ml of solution A and brought to 1 liter) and poured into a 250 ml flask, where it was swirled rapidly for a few minutes under a stream of nitrogen, stoppered, and placed at 4C. At intervals of 2 days, the pH was adjusted to 7.8 with tris buffer.

After 2 or more days of storage in the cold, the preparation was centrifuged for 60 minutes at 16,000 rpm in a Sorvall RC2-B centrifuge (type SS-34 head) at 4C. The supernate was poured into 10 volumes of cold acetone (-20C) and left at -20C until a white precipitate was formed (5 to 10 minutes). This material was centrifuged in the cold in large stainless steel centrifuge tubes in the Sorvall RC2-B centrifuge. The acetone-supernate was discarded after each centrifugation until all the sedimented material had been collected. This sediment was washed once with acetone, then once with anhydrous, peroxide-free diethyl ether (J.T. Baker Chemical Company, Phillipsburg, New Jersey), and dried <u>in vacuo</u> at room temperature using a 125 ml side-arm Erlenmyer flask connected to a vacuum. This Triton extract was ground in a mortor, weighed, and suspended in about 40 ml of 0.85

per cent saline. The dry weight of this material was 0.68 grams. The saline suspended material was mixed for 60 minutes at 4C, then centrifuged for 20 minutes in the cold at 16,000 rpm. The supernate was collected and the sediment resuspended in about 12 ml of PBS, pH 7.2, but without the 60 minute mixing at 4C before centrifuging. These pooled extracts were stored frozen in 10 to 15 ml aliquots and designated Triton soluble lipoprotein (TSL). Before use in chemical, analytical, or biological experiments, the TSL preparation was thawed at room temperature and centrifuged at 100,000 X g for 1 hour in a Beckman Model L-2 preparative ultracentrifuge with the type 50 Titanium head. The supernatant material, except for approximately 1 cm at the bottom of the tube, was concentrated in the cold by negative pressure dialysis using 10 mm flat dialysis tubing (LaPine Scientific Company, Berkely, California). The bottom 1 cm of the supernate plus the small amount of precipitate were discarded.

An early attempt was made to digest lyophilized portions of the saline insoluble preparation, i.e., the sedimented material obtained after extraction with saline and PBS, with lyophilized Crotalus adamanteus venom (Russell Reptile Institute, Silver Springs, Florida). However, this procedure was abandoned due to complications concerning the potency of the venom. This procedure may also be found in the article by Ogburn et al. (95).

Isolation of Soluble Lipoproteins from Normal Tissues

Control cell preparations were derived from liver and/or spleens from groups of 10 to 20 A/Jax mice that ranged in age from $1\frac{1}{2}$ to 7 months, with each group of mice being approximately the same age and weight. The mice were sacrificed by cervical dislocation and the liver and/or spleens were removed and placed in the cold in a petri dish containing a small amount of PBS, pH 7.2. These organs were minced and screened through an 80 mesh, stainless steel wire (Michigan Wire Cloth, Detroit, Michigan) into cold PBS. These single cell suspensions were then placed into tubes and treated in the same manner as described for SaI ascites tumor. The final ILP preparation of these tissues was not frozen and used for immunization, but treated directly with Triton X-100 to obtain the TSL material. Much lower yields were obtained from normal tissues than from SaI. For this reason, in some instances, the precipitate obtained after the 2 hour extraction for ILP preparation was resuspended in 12.5 volumes of cold distilled water, extracted again for 2 hours, and prepared as before in an attempt to improve yields. This ILP preparation was added to the original, and the combined material was resuspended in PBS and dialyzed.

Analytical Methods

Protein concentrations were determined by the method of Lowery et al. (68) with BGG as a standard. Spectrophotometric measurements were made on a Coleman 124 spectrophotometer. Lipid content was determined by a modification of the gravimetric technique of Enternman (24). In this method, a lyophilized and pre-weighed 1 gram sample is mixed with 25 ml of chloroform-methanol (2:1). The mixture is stirred vigorously for three minutes and then placed on a rotator overnight at room temperature. The preparation is filtered through a pre-dried and weighed sintered glass filter. The filter is allowed to dry at 80C overnight and then weighed again. The difference between the weight of the untreated preparation and the weight of the lipid-free preparation was used to establish the lipid content of the TSL preparation.

Glucosamine content was determined by the method described by Ashwell (3) with glucose as a standard and the anthrone determination of carbohydrate content was preformed according to the method of Morris (87).

Ultracentrifugation Studies and Disc Gel Electrophoresis

Sedimentation velocity experiments were performed at 20C in a Beckman Model-E analytical ultracentrifuge with the assistance of Dr. John Robbins of the Montana State University Chemistry Department.

The sedimentation coefficient was corrected to $S_{20,w}$. The protein concentration of the TSL preparation under study was approximately 10 mg/ml.

TSL preparations were electrophoresed on $7\frac{1}{2}$ per cent polyacrylamide gels with tris-glycine buffer, pH 8.4, at 5mA per gel for 3 hours. The technique used was essentially that as described by Davis (20). The gel consisted of small pore solutions 1 and 2 only, as referenced. Bromphenol blue, 0.05 per cent, was used as a marker. After electrophoresis, the gels were fixed and stained with 0.025 per cent Coomassie Blue (5 parts methanol, 1 part acetic acid, 5 parts water, 0.025 per cent stain) for 4 hours and destained with a 7.5 per cent acetic acid-5 per cent methanol mixture at 50 volts. The gels were then examined for homogeneity.

Electron Microscopy

The SaI-ILP preparation was centrifuged at low speeds in preparation for ultrastructural observation. The precipitated specimen was fixed for 1 hour with 2.5 per cent gluteraldehyde in 0.1M potassium phosphate buffer, pH 7.3, followed by fixation in 1 per cent osium tetroxide, also in the same buffer. The washing buffer solution was 0.01M potassium phosphate buffer, pH 7.3. Dehydration was performed by sequentially placing the specimen in the following solutions: (i) 20 per cent acetone (5 minutes), (ii) 70 per cent acetone

(24 to 72 hours), (iii) 100 per cent acetone (15 minutes), (iv) 100 per cent propylene oxide (15 minutes). After infiltration for 1 hour with equal volumes of embedding plastic and propylene oxide, embedment was performed using Araldite epoxy resin, DDSA hardener, and BDMA accelerator. Polymerization occurred after transfer of the embedded specimen to a 60C oven for 24 hours. Ultrathin sections of the SaI-ILP preparation were made on a Reichert Om U2 ultramicrotome utilizing glass knives cut with an LKB 7800A knife maker and floated onto copper grids. Post section staining was performed with 2 per cent aqueous uranyl acetate for $1\frac{1}{2}$ hours followed by treatment with Reynold's lead citrate solution for 3 to 5 minutes. Final sections were observed and photographed using a Zeiss EM9A electron microscope. For more details on ultrathin sectioning techniques, see Wischnitzer (115).

Sarcoma I Tumor Challenge

Challenge was made with live SaI ascites tumor cells which had been removed aseptically from a tumor bearing A/Jax mouse with a 5 ml syringe equipped with a 22-guage needle. The tumor cells (0.5 to 1.0 ml) were placed in a cold centrifuge tube which contained 9 ml of PBS, pH 7.2, and 1 ml of Alsever's solution. The cells were washed 3 to 4 times and then resuspended in 5 to 10 ml of PBS. Cell counts were performed with a hemocytometer, using a 2 per cent acetic

acid-methylene blue solution as the diluting fluid. Mice were challenged intraperitoneally (i.p.) with 1000 SaI cells contained in 0.5 ml.

Injection of Mice

The antigenic activity of the Triton solubilized SaI preparation (SaI-TSL) was ascertained by determining its capacity to either decrease or prolong the survival time of A/Jax neonatal and adult mice after challenge with SaI tumor cells. Mean survival times (MST) were recorded as the average number of days of survival for each group post-challenge.

Adults

A total of 40, 3 to 5 month old adult female A/Jax mice weighing approximately 20 grams each, were used in two identical experiments. The mice were divided into 4 groups. Each group received a single immunizing intraperitoneal (i.p.) injection of one of the following on day 0: (i) 900ug protein/ml of SaI-TSL (1 ml), (ii) 90ug protein/ml of SaI-TSL (0.1 ml), (iii) 20ug protein/ml of liver-TSL (1.0 ml), (iv) saline (1.0 ml). At days 7, 13, and 19, 36 of the mice in these two experiments were immunized i.p. with 0.4 ml of the SaI-ILP preparation to provide a secondary respone. Four of the saline injected mice remained unimmunized. On day 26, all 40 mice were challenged with SaI tumor as previously described.

In a third experiment, 40 adult A/Jax mice, male and female, aged $1\frac{1}{2}$ to $6\frac{1}{2}$ months were divided into 4 groups. The mice in each group recieved an i.p. injection of one of the following on day 0: (i) 900ug protein/ml of SaI-TSL, (ii) 90ug protein/ml of SaI-TSL, (iii) 90ug protein/ml of liver and/or liver-spleen TSL, (iv) saline (0.4 ml). At days 7, 13, 19, and 25, 25 of the 40 mice in these groups were immunized i.p. with 0.4 ml of the SaI-ILP preparation. On day 32, all 40 mice were challenged with SaI tumor as previously described.

Neonates

Only litters containing 4 or more mice were initially utilized. A total of 52 neonatal A/Jax mice were divided into 3 groups. Each litter born was again divided into 2 groups, consisting of one control group and one experimental group. Each group of mice received an i.p. injection of one of the following on days 0, 1, and 2: (i) 90ug protein/ml of SaI-TSL, (ii) 20ug protein/ml of liver and/or liver-spleen TSL, (iii) control volumes of saline. On days 7, 8, and 9, the same groups of mice received one i.p. injection of 2 times the dose of the same material they originally received on day 0. On days 14, 15, and 16, the same groups of mice received one i.p. injection of 3 times the dose of the same material they originally received on day 0. To determine whether tolerance or immunity followed

pretreatment with soluble SaI-TSL, 34 of the 52 mice were immunized i.p. with 0.4 ml of the SaI-ILP preparation on days 30, 36, 42, and 48. The mice were then challenged on day 52 with SaI tumor as previously described.

RESULTS

Yields of SaI and Control Preparations

Table I compares the yields obtained from SaI and liver-spleen preparations at various stages in the isolation procedure as outlined in Figures 1 and 2. Overall, the extraction of liver-spleen preparations yielded 54.6 per cent less soluble material than was obtained from a comparable mass of SaI.

Extracting the liver-spleen ILP preparation twice resulted in an average increase in dry weight of the TSL preparations of about 36 per cent. The double extraction was not, however, considered feasible for SaI-ILP preparations.

Chemical Properties

A typical ILP preparation of SaI was found to have an average protein concentration of 4600ug/ml, 512ug/ml of RNA, 22.5ug/ml of carbohydrate, and no DNA or hexosamine, as determined by Jutila (46), unpublished results. He found that a liver-ILP preparation contained an average protein concentration of 1100 to 1200ug/ml, 150ug/ml of RNA, 10ug/ml of carbohydrate, 368ug/ml of DNA, and no hexosamine.

SaI-TSL preparations were found to contain much less protein than ILP preparations. The average protein content in an unconcentrated preparation of SaI-TSL was approximately 850ug/ml. In addition, the SaI-TSL preparation consisted of 52ug/ml of carbohydrate, and no hexosamine. Liver and liver-spleen preparations were found to contain

Table I. Comparison of yields obtained from SaI and liver-spleen preparations at various stages in isolation.[a]

Preparation	No. of mice	No. of extractions	ILP-PCV (ml)[b]	Extracted volume (ml)[c]	TSL dry wt. (grams)[d]
Liver-spleen	16	1	18.0	192	0.1812
Liver-spleen	17	2	17.5	212	0.3004
Liver-spleen	17	2	17.3	213	0.3047
Liver-spleen	16	2	17.0	212	0.2448
SaI	18	1	29.5	392	1.0131
SaI	21	1	26.0	335	0.8829
SaI	16	1	23.0	266	0.6790
SaI	14	1	16.5	203	0.4782

[a] See Figures 1 and 2 for isolation stages.

[b] Original packed cell volume of immunizing lipoprotein preparation.

[c] Volume of ILP preparation after 2 hour extraction with water at 4C.

[d] Dry weight of Triton soluble lipoprotein preparation obtained after grinding in a mortor.

approximately 20ug/ml of protein, 6ug/ml of carbohydrate, and no hexosamine. The chemical composition of these various materials is summarized in Table II. In addition, the percentage of lipid determined in a SaI-TSL preparation was 42.5 per cent, whereas none could be detected in a liver-TSL preparation.

These preparations were found to be relatively stable to freezing for 2 to 3 months. However, a protein determination made on a SaI-TSL preparation at 5 months after freezing showed a 62 per cent decrease.

Electron Microscopy

Ultrastructural analysis of the SaI-ILP preparation revealed what appeared to be an abundance of ribosomes, lipid bodies, and membranous fragments. Figure 3 is an electron micrograph of this specimen magnified 58,000 times showing these various elements. These findings lend support to the chemical data in that the SaI-ILP preparation was found to contain RNA (ribosomes), and presumably membrane protein and lipid. Electron microscopy failed to identify discrete structures in SaI-ILP preparations.

Ultracentrifugation and Disc Gel Electrophoresis

Sedimentation velocity analysis of the SaI-TSL preparation revealed a single broad sedimenting boundary after centrifugation for 89 minutes, indicating a heterogenous material. Figure 4 shows

Table II. Chemical composition of ILP and TSL preparations.

Preparation	Protein (ug/ml)	Carbohydrate (ug/ml)	Hexosamine (ug/ml)	RNA (ug/ml)	DNA (ug/ml)
SaI-ILP	4600	22.5 (2.25%)[a]	0	512	0
Liver-ILP	1200	10.0 (2%)[a]	0	150	368
SaI-TSL	850	52.0	0	ND[b]	ND[b]
Liver and/or liver-spleen-TSL	20	6.0	0	ND[b]	ND[b]

[a] Per cent of dry weight.

[b] Not determined.