EFFECT OF *ULEX EUROPEUS* SEED EXTRACT UPON THE LYMPHOCYTE METABOLISM

by

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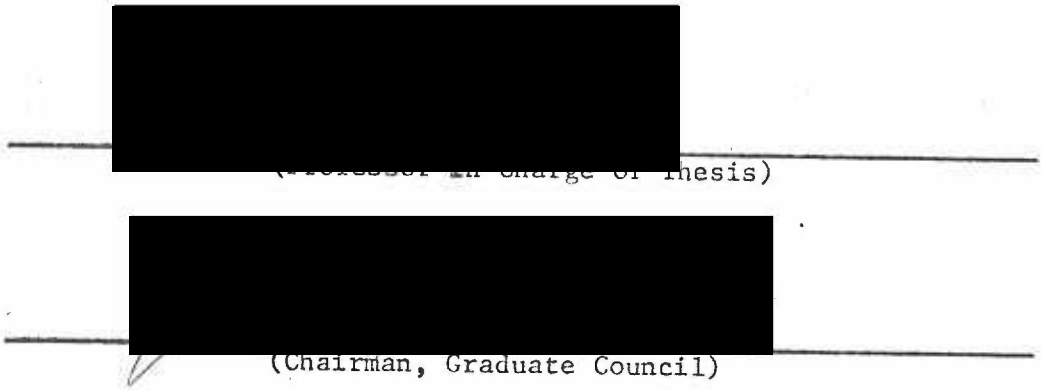

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INTRODUCTION

Original observation

This thesis is concerned with *in vitro* transformation, proliferation and function of normal and malignant human lymphocytes in the presence of a crude extract of *Ulex europeus* seeds. The work is based on the original observation that a trace amount of this extract, when incubated with lymphocytes, impairs metabolism as reflected by an inhibition of DNA and protein synthesis. This inhibition appears to be reversible, with a return of metabolic function when the extract is removed by washing.

Survey of literature

In recent years, some insight has been gained into the complexity of the molecular events which regulate cell metabolism. This in turn has expanded our understanding of the cellular structure. There are several important reasons for the rapid progress made in this area. These include the improvements in the sensitivity and discrimination of analytical procedures, an increasing availability of drugs inhibiting specific reactions in the metabolism of macromolecules, and the development of a "reporter molecule" concept. The close relationship between the nucleus and the membrane of a cell has been clearly shown by many investigators (1). Receptors located on the membrane appear to represent a "medium" between the intracellular compartment and the cell environment.

Lectins

Lectins have assumed great importance as a biological probe for glycoprotein analysis in the study of cell membranes. Several survey articles are available which summarize the history of lectins (2, 3, 4). Basically, lectins are glycoproteins that possess the remarkable ability to agglutinate erythrocytes and other types of cells. Such agglutinins are found predominantly in the seeds of plants, in particular the legumes. However, they are also present in lower vertebrates such as fishes (5) and invertebrates such as mollusks (6). Plant agglutinins are commonly referred to as phytohemagglutinins. However, as cell-agglutining protein also occurs in organisms other than plants, the term "lectins" proposed by Boyd (3) appears more suitable. In addition to their ability to agglutinate red blood cells, which makes their detection easy, lectins exhibit a host of other interesting and unusual biological and chemical properties. The early history of the plant agglutinins has been reviewed by Bird (2). Stillmark reported the hemagglutinin activity of extracts of the castor bean (Ricinus communis) in 1888. Landsteiner noted that these extracts did not equally agglutinate the blood of different species and compared this specificity with that of antibodies of animal origin (7). For several decades it was assumed that plant hemagglutinins were nonspecific in their erythrocyte agglutining action. The first plant extract found to contain blood-group specific agglutining capacity was Phaseolus limensis. These lima beans were ground and extracted with salt solutions. The resulting extract strongly agglutinated erythrocytes of some

human individuals, but those of others only weakly if at all (8). In 1948, Renkonen published an account of his studies of 57 species belonging to 28 genera. He reported Vicia cracca extracts to be specific for the blood-group A antigen and Laburnum alpinum, Cytesus sessilifolius and Lotus tetragonolobus to be specific for the H-blood group factor (9). Over two-thousand species have now been screened for such activity (10). The most common activities are anti-A_1, anti-H, anti-N and anti-M. An apparently new specificity was found in Arachis hypogaea (11). This factor called "peanut factor," agglutinated an antigen present in almost all human erythrocytes and was found to be anti-Gy specific. More recently, Bird and Wingham have found an agglutinin in extract from seeds of Clerodendron trichotonum that seems to have a specificity within the Rh blood group (12). The specific lectins preferentially agglutinate human erythrocytes of a given blood type and form precipitates with corresponding soluble blood group substances. These type specific lectins are inhibited best by saccharides which serve as part of the immuno-determinant of the corresponding blood group substance. Indeed, the first information concerning the role of sugars as determinants of blood group specificity was obtained in 1952 by Watkins and Morgan. These investigators studied type A-specific agglutinins of plant origin (Vicia cracca and Phaseolus limensis) and type H (O)-specific agglutinins from the serum of the eel (Anguilla, anguilla) and the seeds of Lotus tetragonolobus (13). Since N-acetyl-D galactosamine specif-

ically inhibited type A-specific lectins, Watkins and Morgan concluded that this sugar serves as a determinant of human blood group A specificity. Similarly, the agglutination of blood group O cells by the O (H)-specific lectins was best inhibited by α-L fucopyranoside, indicating that the α-L fucosyl residue is a determinant of H (O) specificity. Both conclusions have been fully substantiated in subsequent studies (14). Among lectins which are not specific for human blood groups, a few are known to be highly specific with respect to the binding of saccharides (15).

A more complex pattern of specificity emerges when the agglutination of erythrocytes, as well as other cells from various animals, is examined and compared with the sugar specificity of these lectins (16). Lectins that do not act on erythrocytes but will agglutinate sarcoma-180 cells (17) or leukocytes (18) are present in the red kidney bean and appear to be distinct from the hemagglutinins found in the same bean.

Certain lectins are mitogenic in that they can stimulate the transformation of lymphocytes from small "resting" cells into large blast-like cells which may ultimately undergo mitotic divisions (19, 20, 21). The recent demonstration of lectins specific for tumor cells (22, 23) has led to a great surge of interest in these compounds, especially among those workers engaged in cancer research. Some lectins preferentially agglutinate mammalian tissue culture cells that have been transformed by oncogenic viruses or by chemical carcinogens, as well as spontaneously transformed cells. These and related findings

indicate that the surface of a transformed cell differs from that of its untransformed counterpart and raise the hope that studies with lectins may lead to a better understanding of neoplasia. In addition, they have prompted investigations to use lectins as inhibitors of growth of malignant cells in vitro and in vivo (24) and lectin binding synthetic polymers for the immunization of mice against tumors (25).

Ulex europeus

In 1952, Cazal and Lalaurie (26) reported that crude extracts of several leguminous seeds, including Ulex europeus, had an anti-H specificity. They could be used for the determination of subgroups of various A and AB erythrocytes. The importance of this discovery became more apparent with improved understanding of the biochemical pathway leading to the presence or absence of A, B, H and Lewis substances on the red cell (14). It was shown that all normal individuals possess a precursor blood group substance which is acted on by products of a gene called H and converted to H substance. This in turn is acted on by the products of the respective A, B and O genes. The A and B genes control conversion of the H substance to A and B substances, leaving a variable amount of H substance unconverted. When A and B genes are absent (type O), the H substance remains intact. In rare instances, an individual lacks the H gene and the precursor mucopolysaccharide remains unchanged. Hence the A and B genes cannot express themselves and the cells are not agglutinated by anti-A, anti-B or anti-H sera. These individuals are classed as "Bombay" phenotype. Their cells are designated O_h. The frequency of this phenotype is

very low. Only 20-30 examples have been reported (27). Approximately 80 percent of the population secrete the soluble blood group substances into various body fluids, with A, B and H specificities corresponding to each individual's blood type; the remainder termed "non-secretors" do not. Thus a type A "secretor" has A and H substances present in body fluids such as saliva, gastric juice, serum, seminal fluid, tears and breast milk. Ulex europeus, having the ability to bind to H substance, can discriminate secretors from non-secretors. The lectin is mixed with saliva from the individual and thereafter tested against type O cells. A decrease in the original agglutination titer indicates the presence of H substance in saliva and the individual is termed a "secretor" (28). Recent studies have shown that there are two kinds of anti-H phytohemagglutinins in the seeds of Ulex europeus (29, 30). One was found to be inhibited by a methyl-pentose, L-fucose (3) and the other was inhibited by salicin (30).

Ulexine

Ulex europeus, like many other plants of the same family, have been utilized for their medicinal value (31), since the beginning of this century. The ground seeds yield an alkaloid material having a common denominator of being nicotine-like. These are referred to as lupine alkaloids or under the name of the specific extracted plant, i.e., ulexine from Ulex europeus, cytisine from Cytisus sessilifolius. Such compounds have been frequently employed in many folk remedies as antitussive and antipruritic agents (32).

ABH antigens in human lymphocytes

There have been many studies to delineate the presence of red blood cell antigens in leukocytes. Due to a lack of uniformity in methodology, results have been controversial. However, the cross-reactivity of leukocytes and red cell antibodies in mixed agglutination (33) and the wide distribution of ABH substances in human tissues (34) led investigators to use more sophisticated techniques. The use of radioactive labeled anti-A and anti-B has shown evidence for the presence of A and B antigens on human leukocytes (35). In addition, the presence of water-soluble ABH blood group antigens was demonstrated by reacting disrupted lymphocyte extracts with their appropriate type specific antibody in single gel immunodiffusion tests (36). The failure to obtain analogous results with red cell extracts is consistent with the concept that the greater portion of the ABH antigens in erythrocytes are extractable only by lipid solvents (37). The possibility of non-specific absorption of soluble ABH antigens from the plasma was eliminated by the observation that these antigens were not related to the secretor status of the leukocyte donor and could not be transferred to leukocytes by incubating with plasma of a strong secretor. The amount of blood group substances on lymphocytes has been estimated to approximate one-fiftieth of the amount present on red cells (38).

The lymphocyte

It is well established that mammalian lymphocytes represent two major functional classes, the thymus-dependent T-cells and the thymus

independent B-cells (39). Each of these classes can probably be further divided into subpopulations, differing in particular immunological activity or stage of development (40).

Antigen recognition and immunologic memory are associated with the small lymphocytes. These cells are ultimately derived from bone marrow precursors (41). Bone marrow stem cells differentiate to form the two distinct lymphocyte populations: the thymic-dependent T-lymphocytes which are responsible for the initiation of cell-mediated immunity, and the thymic-independent (bursa-equivalent) B-lymphocytes which are responsible for the initiation of humoral antibody synthesis.

T-lymphocytes

The differentiation of embryonic stem cells to thymocytes and eventually to peripheral T-lymphocytes is a complex process only partially understood. However, accumulated data from experiments performed in chicken and mice suggest that the thymus gland plays a major role in programming the entire immune system. In addition to the obvious morphological events which take place within the developing lymphoid cell-line of the thymus, i.e., the transition from large basophilic cell to small lymphocyte, profound changes also take place in the representation of cell surface alloantigens. Although an unknown number of thymic lymphocytes may die *in situ*, cell marker experiments have demonstrated that some of them migrate to peripheral lymphoid tissue where they are appropriately termed "thymus-derived" or T-lymphocytes. These cells form the greater part of the

circulating pool of small lymphocytes. Their role in cell-mediated immunity has been extensively investigated in various model systems of delayed hypersensitivity (42). It has been shown that T-cells contain antigen-reactive sites located on the membrane and are therefore capable of antigen recognition. On contact with antigen, T-lymphocytes enter into a poorly understood series of cellular events which ultimately result in the release of soluble factors. These materials induce tissue changes considered to be characteristic of delayed type hypersensitivity (43). In addition, it has been observed and confirmed that T-cells somehow assist B-cells to produce antibody (44). The observation made by Greaves and Janossy (45) that phytohemagglutinin (PHA) selectively stimulates thymus-dependent lymphocytes, supplies a useful marker for these cells.

B-lymphocytes

B-cells are derived in birds from the bursa of Fabricius. Mammalian lymphocytes of thymic-independent origin are similarly referred to as B-lymphocytes. However, formal proof that they are analogous to avian bursal lymphocytes is still needed. In the rabbit, stem cells of hemopoietic tissue must migrate to gastrointestinal lymphoid tissue for induction of differentiation along the plasma cell-line (46). A number of markers for B-type lymphocytes are now available. These cells have surface immunoglobulins which are readily detected by fluorescein-conjugated anti-heavy and anti-light chain sera (47). In addition, it has been demonstrated that B-lymphocytes

carry a receptor for antigen-antibody-complement complexes (48). Surface alloantigens specific to B-cell have also been shown in the mouse (49).

Distribution of B and T-cells in the human

Staining of lymphoid cells with immunofluorescent reagents specific for immunoglobulin determinants has been employed to quantitate the percentage of B-cells in human lymphoid tissue. Approximately 28% of human peripheral lymphocytes are coated with immunoglobulins and are presumably B-type lymphocytes. Tonsillar tissue and lymph nodes have a greater percentage of lymphoid cells bearing surface immunoglobulins than the peripheral blood. Thymocytes do not have demonstrable surface immunoglobulins employing the usual fluorescent techniques (50). The majority of peripheral blood lymphocytes obtained from patients with chronic lymphocytic leukemia have been shown to bear immunoglobulins on their cell surface (51).

Nature and scope of lymphocyte cultures

The demonstration by Harrison in 1907 that cells could normally function in vitro has been generally accepted as marking the true beginning of tissue culture. This has incidentally illuminated the potentialities of experimental methods using surviving cells in vitro (52). Success in this field suggested the possibility that cells might be grown similar to that of microorganisms (53). The possible use of large numbers of cells in metabolic studies was a particularly intriguing thought and was developed by several investigators. Blood was one

of the tissues which attracted a great deal of attention due to its availability and the polymorphism of its elements. Unfortunately, the lymphocyte was neglected. This cell was considered to be unsatisfactory because of its small size and its short survival *in vitro*. It was thought that small lymphocytes were nothing more than end-stage cells, incapable of transformation (54). This viewpoint was modified by the studies of Hungerford (55) in 1959 and Nowell (19) in 1960 and confirmed by others (20, 21). They demonstrated that phytohemagglutinin (PHA), a saline extract derived from beans of the genus *Phaseolus* was capable of transforming small lymphocytes into primitive dividing cells. Such studies created tremendous interest and attracted many workers to the field of lymphocyte physiology. Several survey articles are available which summarize the history of lymphocyte transformation (56, 57).

It is now well recognized that several substances called mitogens induce striking morphological changes in human peripheral blood lymphocytes. Large blast-like cells appear in cultures that previously contained predominately small lymphocytes; these transformed cells progress to division. Nonspecific mitogens induce transformation of lymphocytes obtained from individuals who have not been sensitized. Examples of nonspecific mitogens include phytohemagglutinin (PHA) (18), pokeweed (an extract of *Phytolacca americana*) (58), staphylococcal filtrate, streptolysin-S (57), purified protein derivative of tuberculin (PPD) (59), antiglobulin serum (60), antigen-antibody complexes (61), mercuric chloride (62), and sodium periodate oxidation (63). These nonspecific stimuli induce most human lymphoid cells in culture to

incorporate tritiated thymidine in newly synthesized DNA. In addition, such stimulated cells may progress to replication.

Other mitogens are termed specific and require previous *in vitro* contact with lymphocytes. They are stimulatory to a more restricted lymphocyte population. Smaller amounts of tritiated thymidine are incorporated with the specific reactions than with nonspecific stimulation. *In vitro* sensitivity to specific stimuli have been demonstrated with bacterial toxoids, such as tetanus and diphtheria, with penicillin and other drugs, with viruses such as vaccinia (57) and with allergens such as ragweed pollen (64) and timothy (65). These agents have been associated with both cellular and humoral antibody mediated hypersensitivity. Antibody to heterologous lymphocytes called anti-lymphocyte serum (ALS) has also been shown to be mitogenic in the absence of complement (66). The finding that ALS is mitogenic suggested to Coulson (67) that special recognition sites are located on the surface of lymphocytes. It is suggested that ALS recognizes and attaches to heterologous sites. Thereby, it triggers the chain of events which is called transformation.

Bain *et al.* (68) have shown that when lymphocytes from two unrelated individuals are cultivated together, transformation occurs. Studies with monozygotic twins and non-related individuals indicated that the degree of transformation was related to the genetic differences between lymphocytes of the donors. Mixed lymphocyte culture (MLC) is now used as a histocompatibility test. Studies with this test system suggest that the degree of stimulation in MLC is inversely related to

the survival time of graft between the same individuals (69).

Circulating lymphocytes from many animals have been found to respond to PHA and other mitogens. The poor survival of non-human lymphocytes *in vitro* present technical difficulties. However, human peripheral blood lymphocytes may survive *in vitro* up to three weeks (57). Cells other than circulating lymphocytes have been used in transformation experiments and these cells show varying degrees of responsiveness. Lymph nodes, spleen, thymus, appendix, and tonsil lymphocytes have been successfully cultured and used (57).

Lymphocyte transformation is a phenomenon of exceptional importance. It requires consideration of the lymphocyte as a metabolically active cell, rather than viewing it simply as an immunologic unit. Accordingly, such studies have accelerated the entry of immunology into cell biology. It is a revealing model system for studying the fundamentals of cellular activation and differentiation (70).

Lymphoid cell-lines derived from human peripheral blood have been established in apparently permanent cultures. Lymphocytes from normal individuals (71, 72) and from subjects with various benign (73) and malignant (74, 75) lymphoproliferative diseases have been successfully cultured. Considerable numbers of these cell-lines have been maintained for many years of continuous passage, without loss of viability. They grow as free-floating pleomorphic forms. They present a fundamental lymphoid character resembling the immature "blast-like" transformed cells seen following the *in vitro* stimulation of peripheral small lymphocytes by phytomitogens and antigens (76). These cells, in

general, maintain the human diploid chromosome number and appear to retain the genetic phenotype of the host. Intensive study in many laboratories has confirmed the versatility of these cell systems. They manifest stem cell potentials and are capable of activating and synthesizing a variety of products which are of biologic significance in the responses of the immunocompetent host. Immunoglobulins, mediators of cellular immunity, interferon, components of complement, enzymes and soluble histocompatibility antigens are among the known materials synthesized and released into the supernatant culture fluid (77). The remarkable proliferative and synthetic potential of these cell systems make them useful models for the detailed analysis of lymphoid functions and facilitate the study of drugs and naturally occurring substances which influence cellular metabolism. The availability of these lymphoid cell-lines supply an ideal in vitro model of cell replication and differentiation, and facilitate the isolation and precise characterization of human lymphoid products associated with humoral and cell mediated immunologic responses. What has been said about the erythrocyte concerning its accessibility and its abundance can now be applied to the lymphocyte. This cell is now simple to study and the system is generous with dividends.

Study plan

Answers were sought to the following questions:

1. Is the inhibition induced by Ulex europeus seed extract limited to the differentiation of small lymphocytes to lymphoblasts?

2. Is proliferation of lymphocytes, such as seen in mixed lymphocyte reaction or permanently maintained cell-lines, inhibited?
3. Is only DNA synthesis affected by Ulex europeus seed extract?
4. Does the inhibition reflect a cytotoxic action of the crude extract of Ulex europeus seeds?
5. Are only peripheral blood lymphocytes affected by the seed extract? Do thymocytes and tonsillar lymphocytes respond in the same way?
6. Are B-lymphocytes and T-lymphocytes equally affected?
7. Is there a difference in the inhibition of normal and malignant lymphocytes?
8. What causes this inhibition? Is it the anti-H hemagglutinin present in extracts of Ulex europeus seed? Is it cytesine, a naturally occurring alkaloid material present in many leguminous plants?

In order to answer these questions, an optimal tissue culture system was established for human lymphocytes. Phytohemagglutinin (PHA) and pokeweed mitogen (PWM) were employed as nonspecific mitogens. In this tissue culture system, mixed lymphocyte culture was utilized as specific mitogens. A series of experiments were carried out in which spontaneous DNA synthesis of freshly explanted, non-stimulated lymphocytes was measured after 3 hours of culture with and without the lectin extract. The observation was made that reactive

lymphocyte blastogenesis enhanced the inhibitory effect of *Ulex europeus* seed extract. Patterns of lymphocyte reactivity were observed which suggest a speculation basis for *in vitro* cellular inhibition.

MATERIALS AND METHODS

I. Ulex europeus seed extract

Ulex europeus, better known as gorse, is a stout, rigid evergreen shrub with bright yellow flowers. It was introduced from Europe to the United States for its ornamental properties. It is classified as a Leguminosae. Its seeds are widely used in immunohematology due to their anti-H agglutinin content.

The procedure described by Moore et al. was used with minor modifications for extraction of the seeds (78). Briefly, 50 grams of Ulex europeus seeds (S. B. Penick & Company, New York, N. Y.) were ground for one minute in a Waring Blender. Isotonic saline (150 ml) was added and thirty minutes later, when the seeds had absorbed the fluid, the mixture was further diluted with 100 ml of Hendry's iso-osmotic phosphate buffer, pH 6.8, and 100 ml of iso-osmotic glycine solution (2.2 grams per 100 ml of distilled water). The seeds were mixed briefly in the blender and incubated at room temperature for three hours. The suspension was centrifuged at 34,800 X g for 30 min. and the supernate heated at 60°C for 30 min. until flocculation occurred. Floccules were removed by centrifugation 27,000 X g for 30 min. and the cloudy supernatant was clarified by filtration. The clear solution referred to as the crude extract was sterilized through a Millipore filter, 0.45 micron (Millipore Corporation, Bedford, Mass.) and stored at 4°C. This extract was used throughout the experiments unless otherwise indicated. It was added directly to the cell cultures at a

concentration of 1% and 5% (V/V). Each ml. of crude extract had a dry weight of 48 mg.

Heated extract of Ulex europeus

The crude extract of Ulex europeus was heated at 100°C in a water bath for 30 min. The cloudy solution was clarified by centrifugation at 27,000 X g for 30 min. and the supernate stored at 4°C until use.

Dialyzed extracts of Ulex europeus

An aliquot of the crude extract of Ulex europeus was dialyzed in the cold for 48 hr. against Tris-HCL buffer at pH 7.2 (500 ml. changed twice). This procedure was performed to eliminate glycine and other small molecules which could inhibit lymphocyte DNA synthesis.

Water soluble fraction of Ulex europeus seed extract

Additional samples of the crude extract were dialyzed against distilled water for 48 hr. in the cold. The contents of the dialysis bag became cloudy and precipitation occurred. This material was clarified by centrifugation at 27,000 X g for 30 min. and the supernate dialyzed against Tris-HCL buffer at pH 7.2 to re-establish the ionic strength of the solution.

Trichloroacetic acid fraction of Ulex europeus seed extract

The crude extract of Ulex europeus seed was mixed with trichloroacetic acid at a final concentration of 5% (V/V) to precipitate protein. After incubation in the cold for 30 min. the suspension was centrifuged at 27,000 X g for 30 min. The supernate was dialyzed overnight

against Hendry's isotonic phosphate buffer to remove trichloroacetic acid. This solution was referred to as the trichloroacetic acid soluble fraction.

The precipitate was dissolved in 1 N NaOH and reconstituted to the original volume with Hendry's buffer. Dialysis was performed employing a phosphate buffer and this fraction was referred to as the trichloroacetic acid insoluble fraction. Both solutions were sterilized by passage through a Millipore filter and stored at 4°C.

Ulexine extraction

Since plant alkaloids generally exist as water soluble salts, extraction with acidulated water produces a crude extract which can be tested directly with standard alkaloid precipitating reagents. A purification procedure (79) is required as chromogens and other materials capable of inducing false positive alkaloid tests may be present. This is usually accomplished by the addition of base and subsequent extraction with a water immiscible organic solvent. The procedure used to extract the alkaloid material from Ulex europeus seeds was basically the one described by Ing (80). Ten grams of Ulexeuropeus seeds were ground in a Waring Blender for 5 min. Boiling distilled water, 30 ml., was added and the preparation heated for one hour at 100°C in a water bath. The pulp was further extracted with hot distilled water to a final volume of 150 ml. The mixture was centrifuged at 37,800 X g for 30 min. The supernate was filtered through glasswool to remove floating material and its pH adjusted to 9.0 with 2 N NaOH. This solution was lyophilized. The dry material was

stirred with 75 ml of chloroform at room temperature for 30 min. The chloroform was decanted and saved. The same procedure was repeated with 10 ml of chloroform overnight in the cold. The chloroform extracts were filtered and flash evaporated at 45°C. The residue was washed with absolute ethanol and evaporated to dryness at 45°C. The final residue was dissolved in saline, sterilized through a Millipore filter and stored at 4°C. An aliquot was tested for its alkaloid content by the method of Munier with the use of Munier-Macheboeuf-Dragendorf's reagent (81).

II. Saliva

Saliva from blood group "O" secretor individuals was collected and prepared according to the method of Issitt (82). Neutralization of anti-H activity from Ulex europeus seed extract was carried out by the method of Boyd (28). The neutralized extract was used at the concentration previously described.

III. Titer of anti-H activity of Ulex europeus seed extract

Titration of anti-H activity of Ulex europeus extracts was performed by Anne August, Division of Immunology and Allergy, U.O.M.S., using a panel of A, B, and O fresh human red blood cells. Dilutions were done in normal human AB serum and scores recorded according to the titration method described by Marsh (83).

IV. Lymphocyte sources

Peripheral blood lymphocytes

Blood was obtained from healthy adults of groups "O", "A_1" and "A_2" and from two patients with chronic lymphocytic leukemia. Blood was mixed with heparin (Panheprin, Abbott Laboratories, North Chicago, Ill.) at a concentration of 50 units/ml of blood. The heparinized blood was mixed with dextran, 5% in saline (M.W. 193,000) at a 1:5 (V/V) concentration and incubated at 37°C for 1 hr in order to induce erythrocyte sedimentation. The leukocyte-rich plasma was removed and centrifuged at 200 X g for 10 min. The platelet-rich portion of plasma was discarded and the leukocyte pellet resuspended in Hanks' balanced salt solution (Grand Island Biological Corp., Grand Island, N.Y.) supplemented with 5% (V/V) normal human AB serum. The leukocyte suspension was then ready for lymphocyte purification.

"Bombay" group blood was kindly provided by Dr. K. Sheth, Department of Clinical Pathology, U.O.M.S. The blood was obtained by venesection from a healthy Indian male in Calcutta, India. Heparinized blood, 20 ml., was injected into the upper nylon pad of a Terasaki Lymphocyte Transport Bag (Life Instrumentation, Glenview, Ill.) and then flushed into the lower cotton pad with 15 ml of McCoy 5-A medium (Grand Island Biological Corp., Grand Island, N.Y.). Three days later, the blood-filled tampon was placed in a 50 ml syringe with an additional 10 ml of McCoy medium and squeezed into a test tube. Red blood cells were removed by permitting the diluted blood to sediment for 1 hr. at room temperature. The leukocyte-rich diluted plasma was then ready for lymphocyte separation.

Tonsillar lymphocytes

Tonsils were obtained from children 4 to 12 years of age undergoing tonsillectomy for the usual clinical indications. The tonsils were placed in Hanks' balanced salt solution containing 500 U/ml of penicillin G (Upjohn Company, Kalamazoo, Mi.), streptomycin 500 µg/ml (Pfizer Laboratories Division, New York, N.Y.) and N-2-hydroxethyl piperazine-N-2-ethane sulfonic acid (Hepes) 0.04 M (Calbiochem, San Diego, Calif.). Usually no more than 2 hours elapsed between operative removal of the tissue and the preparation of cell suspensions. Cells were teased into suspension in medium 199 (Grand Island Biological Corp., Grand Island, N.Y.) with a scalpel, transferred to a sterile 16 X 150 mm plastic centrifuge tube with screw cap (Falcon Plastics, Los Angeles, Calif.) and the tissue fragments allowed to settle for 5 min. The medium overlaying the settled debris was transferred to another centrifuge tube, pipetted to achieve single cell suspension and centrifuged at 200 X g for 10 min. at 4°C. The cell pellet was resuspended and washed twice in medium 199 containing antibiotics and a trace amount of human AB serum (84). After the last centrifugation, cells were resuspended in culture medium supplemented with 5% normal human AB serum.

Isolation of non-complement-receptor lymphocytes from tonsillar lymphocyte suspension

In order to separate B-cells and T-cells from tonsillar lymphocytes, the selective adherence of these cells to glass-beads columns

was used. This procedure was based on the observation of Bianco et al. (48) that complement-receptor lymphocytes (CRL) or B-cells adhere preferentially to nylon wool. Experience with gum-coated glass beads demonstrated their effectiveness in this separation procedure (85). The beads (0.2 mm in diameter) were a variety normally used for reflectorizing road signs. They were washed in distilled water, poured into a 1% solution of gum arabic and dried. The gum-coated beads were tightly packed into a Pasteur pipet which was plugged with nylon wool. Such a column was obtained by sharp intermittent tapping of the column during packing. The column was equilibrated with fresh human AB serum before use. Tonsillar lymphocytes (1×10^8) were suspended in 0.5 ml of fresh human AB serum and applied to the column which was then incubated at 37°C for 15 min. The glass-bead column was eluted with 2 ml of pre-warmed fresh human AB serum. The cells obtained in the eluate were washed with veronal buffer saline (VBS) and tested for rosette-forming ability as previously described (86). Almost all of the complement-receptor lymphocytes (CRL) were trapped in the column. More than 80 percent of the non-complement-receptor lymphocytes (NCRL), or T-cells, were recovered in the effluent.

Assay for detecting complement-receptor lymphocytes

The method for the detection of complement-receptor lymphocytes (CRL) was similar to the one described by Lay and Nussenzweig (86).

Preparation of indicator erythrocytes

Sheep erythrocytes (E) were sensitized with antibody (A) and guinea

pig complement (C). Equal volumes of a 5% suspension (5 X 10^8 cells/ml) of sheep erythrocytes (Prepared Media Labs, Tualatin, Ore.) and a 1:500 diltuion of anti-sheep hemolysis (Grand Island Biological Corp., Grand Island, N.Y.) in VBS (pH 7.4) were incubated at 37°C for 30 min. The sensitized cells (EA) were washed twice and resuspended at the original concentration. An equal volume of 1:80 dilution of fresh guinea pig serum in VBS was added and incubated at 37°C for 10 min. Under these conditions there was minimal red cell lysis. After two washings in VBS, a final suspension of EAC was adjusted to a total of 2 X 10^9 cells in 1 ml of EDTA-VBS (0.01 M EDTA in VBS). The EAC so obtained were tested by immune adherence reactions according to standard technique (87) to confirm the presence of C3. They were stored at 4°C.

Detection of complement-receptor lymphocytes

Since tonsillar lymphocyte suspensions contain variable numbers of monocytes and macrophages, even after differential centrifugation (88), generation of rosettes was accomplished in the presence of ethylene diamine tetracetate-trisodium (EDTA): this procedure has been shown to suppress the binding of EAC to macrophages and monocytes (86). Tonsillar mononuclear cells (5 X 10^7) were added to 10 ml of the stock EAC suspension in EDTA-VBS. The mixture was incubated at 37°C for 30 min. with occasional gentle rotation. Total lymphocytes and lymphocytes surrounded by erythrocyte or rosettes (CRL) were counted on a microscope slide. Controls employing aliquots of the same mononuclear suspensions incubated with EA, were consistently negative for ability

to form rosettes.

Thymus lymphocytes

Thymus tissue was obtained from children between the ages of 6 months and 9 years undergoing surgical correction of congenital heart defects. Tissue was collected and handled in the same fashion as the tonsillar tissue.

Lymphoid cell line

These cells (Itwazuki strain) were kindly provided by Dr. H. M. Grey, National Jewish Hospital and Research Center, Denver, Colorado. Peripheral blood lymphocytes obtained originally from a normal individual were successfully maintained in culture and have been established as a permanent lymphoid cell line (89). Morphological studies by phase contrast microscopy and after Wright stain demonstrate a cell line with varied features. Cell populations, 10 to 15 microns in diameter, with scanty, deeply basophilic cytoplasm and indented nuclei with coarsely clumped nuclear chromatin are present. In addition, there are large, 15 to 24 microns in diameter, cells with an increased cytoplasm to nuclear ratio, light blue vacuolated cytoplasm with occasional azurophilic granules and a nucleus with finely reticulated chromatin material and large nucleoli. These cells are characterized by a spontaneous high mitotic rate. Extracts of Ulex europeus seed were assayed for their capability to stop growth of these cells. Throughout this study cultures were employed from stock maintained in medium RPMI-1640 with fetal calf serum, 15%. RPMI-1640 was obtained from Grand

Island Biological Corp. (Grand Island, N.Y.) and was supplemented with Hepes, 0.04 M, 20% fetal calf serum (Grand Island Biological Corp.) and antibiotics.

V. Lymphocyte micro-culture method

The micro-method described by Sengar and Terasaki (90) was modified to establish a rapid and reproducible test system designed to quantitate the uptake of labeled precursors of DNA, RNA and protein synthesis by human lymphocytes.

Lymphocyte separation technique

Cells from the sources described above were further purified by differential centrifugation over a Ficoll-Isopaque gradient (88). Six ml of a cell suspensions was layered on top of a 2 ml Isopaque-Ficoll solution contained in a sterile 16 X 150 screw cap plastic centrifuge tube. The Isopaque-Ficoll mixture was composed of 10 parts 33.9% Isopaque (Nyegaard and Co., As., Oslo, Norway) and 24 parts of 9% Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden). The lymphocytes were retained at the interphase of the cell suspension and the Isopaque-Ficoll mixture, and were removed with a capillary pipet. The separated cells were washed three times with Hanks' balanced salt solution containing a trace amount of normal human AB serum. During washings they were centrifuged at 200 X g for 10 min at 4°C and the supernatant discarded. After the final wash, the cell pellet was resuspended and pipetted to achieve a single cell suspension in tissue culture medium. This consisted of 20% normal human AB serum inactivated at 56°C for 30

min., culture medium 199 supplemented with L-glutamine (Microbiological Associates, Inc., Bethesda, Md.), sodium penicillin G 100 U/ml, streptomycin 100 µg/ml and Hepes 0.04 M.

An 0.5 ml aliquot of this cell suspension was removed and counted in a Coulter particle counter (Model F, 100 microns = aperture). Viability was estimated by determining the percentage of cells able to exclude trypan blue (91). The criteria for morphological identification of cell types were similar to those of Wintrobe (92). Cells were suspended in human serum and placed in a sedimentation chamber as described by Prochazkova (93). The dried cells were fixed with absolute methanol and stained with Wright stain in the conventional manner.

The original cell suspensions were diluted in tissue culture medium to a concentration of 1 X 10^6 cells/ml.

Lymphocyte culture

An Oxford sampler (Oxford Laboratories, San Mateo, Calif.) was used to transfer 0.2 ml samples of the lymphocyte suspension into polyethylene Microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) previously sterilized under ultra-violet light. Five replicated cultures were set up with each tube containing 0.2 X 10^6 cells in 0.2 ml of tissue culture medium. Reagents were added directly to appropriate tubes either at the start of or during incubation. Tubes were capped and incubated in an upright undisturbed position at 37°C in a humidified atmosphere for a period of 3 hrs to 6 days according to the experiment and test involved. All culture procedures were

carried out under a hood pre-exposed to ultra-violet light.

Quantitation of lymphocyte DNA synthesis

Eighteen hours before harvest, 1 μCi of tritiated methyl-thymidine (New England Nuclear, Boston, Mass., specific activity: 6.7 Ci/mM) in 0.02 ml of medium 199 was added to each tube. Incorporation of the labeled thymidine was stopped by cooling the harvested cultures to 4°C for 30 min. Cells were then centrifuged for 20 seconds in a Beckman Spinco Microfuge Model 152 and the supernatant aspirated and discarded. The cell button was washed two times with iced isotonic saline and the supernates discarded. Then trichloroacetic acid, 0.4 ml of a cold 5% solution, was added to each culture tube and the mixture refrigerated at 4°C for 30 min after vigorous agitation to break up the cell pellet. An insoluble precipitate was formed and isolated by centrifuging the suspension for 1 min. in the Microfuge. The supernatant was aspirated and discarded. Cold absolute methanol, 0.4 ml was added to each tube and the suspension again centrifuged for 1 min. The methanol was aspirated and discarded and the insoluble precipitate dried overnight at 37°C.

The multiple washings and decantation of supernates in this microprocedure was facilitated greatly by employing an aspirating needle designed to minimize loss of either cells or trichloroacetic acid precipitates. A 16 gauge Wintrobe needle was heat-sealed at the tip and opposing holes drilled 2.5 mm. from the sealed end. This modified aspiration needle was attached to a water pump and weak suction

applied. Supernates were then removed by inserting the needle into each tube to the limit set by the stop. A minimal volume of supernate remained. Weight-volume measurement studies demonstrated that the residual supernate plus cell button was less than 3 µl.

Previous studies had shown that the washing procedure described above was sufficient to reduce counts per minute (CPM) to a stable level and so removing cell-free tritiated thymidine present in culture tubes.

The dried trichloroacetic acid precipitate containing incorporated tritiated thymidine was prepared for counting. Culture tubes were cut with a razor blade above the level of the precipitate and tips were placed individually in MiniVials Scintillation Counting Vial (Nuclear Associates, Inc., Westbury, N.Y.). Soluene (Packard Instrument Company, Inc., Downers Grove, Ill.) 0.1 ml, was added and the vials were tightly capped. They were stored overnight at 37°C in the dark. Toluene (scintillation grade) containing POPOP 0.1 gram/liter and PPO 6 grams/liter (Packard Instrument Company, Inc., Downers Grove, Ill.) was added at a volume of 5 ml per vial. The vials were placed in a Beckman Liquid Scintillation Counter (CPM 100) and allowed to equilibrate to light and temperature for 8 to 12 hours. The degree of quenching was determined by an external standard method. Counts per minute (CPM) were not corrected to 100 percent efficiency. Rather, the CPM obtained within a single experiment were evaluated and never compared among several studies. Counting was always performed for a minimum of 5 min. and usually 10 min. periods.

VI. Reactive lymphocyte blastogenesis

T_o test

Freshly prepared cell suspensions from different lymphoid sources were established in culture without stimulation. They were incubated for a 3-hour period and then harvested. Tritiated thymidine was added at the start of incubation and Ulex europeus extract introduced into the appropriate tubes at the usual concentrations. These experiments were initiated in order to evaluate the effect of the crude seed extract on spontaneously occurring DNA synthesis. This process has been described as the T_o of lymphocytes (94).

Lymphocyte transformation experiments

Phytohemagglutinin (PHA) was obtained as the PHA-P powder for 5 ml solutions from Difco Laboratories (Detroit, Mich.). Pokeweed mitogen was obtained as PWM (Backer and Farnes) powder for 5 ml solutions from Grand Island Biological Corp.,(Grand Island, N.Y.). For each batch of mitogen received, a dose-response curve was performed in order to select the concentration initiating optimal responses with human lymphocytes under the standard culture conditions. The stock PHA solution was diluted 1:10 in culture medium and the stock PWM solution used undiluted in the current studies. Each mitogen-stimulated culture contained 0.01 ml of the appropriate mitogen added at the start of incubation. Cultures were then incubated for a period of 90 hours before harvesting.

Mixed lymphocyte culture

One-way stimulation mixed lymphocyte cultures (95) were established

employing lymphocytes obtained from unrelated, ABO compatible individuals. Responding cells were obtained directly from the original cell suspensions and were referred to as A, B, C etc. Stimulating cells were produced by incubating the lymphocytes with 40 µg/ml mitomycin C (Calbiochem, San Diego, Calif.), at 37°C for 30 min. The cell suspension was freedfrom the alkylating agent by centrifuging at 200 X g for 10 min at 4°C and washing three times with medium 199 containing a trace amount of human AB serum. Treated cells were resuspended to the original cell suspension concentration and were referred to as A_m, B_m, C_m, etc. Equal volumes of stimulating and responding lymphocytes were mixed and 0.2 ml samples containing 0.2 X 10^6 cells were dispensed into Microfuge tubes. Control cultures contained treated and untreated cells from the responding individual. These cultures were incubated for a period of 6 days before harvesting.

Viability studies

Dye exclusion

Cell viability was determined by the trypan blue exclusion method. Differential cell counts were performed after the addition of trypan blue to unstimulated cultures containing Ulex europeus extract at the usual concentrations. The procedure was designed to obtain maximal reliability from this test (91, 96). Cells were prepared and incubated as described for DNA synthesis. For five sequential days, cultures with and without seed extract were centrifuged, the culture media decanted and cells suspended in 3 drops of culture medium supplemented

with 20% human serum and 1 drop of 1% trypan blue in distilled water. Five minutes later, stained and unstained cells were counted in a Neubauer hemacytometer white cell counting chamber using phase contrast optics. The percentage of viable cells was determined by the following equation: % viability = [1-(total stained cells/total cells)] X 100.

^{51}Chromium release

Labeling of lymphocytes with ^{51}Cr was accomplished according to the method of Wigzell (97). Peripheral blood lymphocytes to be used in the ^{51}Cr assay were obtained and purified as described for DNA synthesis. Suspensions containing 10^7 cells in 2 ml of culture medium were incubated with 100 µCi of labeled sodium chromate ($Na_2\ ^{51}Cr\ O_4$) (New England Nuclear, Boston, Mass., spec. act. 172 mCi/mg Cr) at 37°C for 30 min with occasional shaking. An equal volume of pre-warmed tissue culture medium was added and the cell suspension centrifuged at 200 X g for 10 min at 4°C. The supernate was discarded; cells were resuspended in 10 ml of iced culture medium and incubated in an ice bath. Thirty minutes later, the cell suspension was centrifuged again and the supernatant discarded. The lymphocyte pellet was resuspended in tissue culture medium at a final concentration of 1 X 10^6/ml. Two sets of cultures without stimulation were established as described previously and Ulex europeus extract was added to the appropriate tubes at the usual concentrations. Each experimental set was composed of control cultures and cultures with 1% and 5% of Ulex europeus seed extract respectively. Five replicated cultures were established for each.

One set was incubated for 3 hours. The cells were sedimented by centrifugation in a microfuge for 10 seconds, and the supernatant removed and stored. The same procedure was performed with the second set of cultures at the end of 24 hours. Supernatants were counted in a well-type scintillation counter with pulse height analyzer (Tri-Carb Scintillation Spectrometer, Packard Instruments). The maximal release of ^{51}Cr was determined by freeze-thawing labeled cells. The percent of ^{51}Cr released was calculated according to the following formula: % release = [CPM released/maximal released] X 100.

Pre-incubation of lymphocytes with Ulex europeus seed extract

Peripheral blood lymphocytes collected and purified by the previously described technique were incubated with Ulex europeus seed extracts at 37°C. Forty-eight and 72 hours later, they were washed three times in medium-199 containing a trace amount of human AB serum. The cells were resuspended in normal tissue culture medium and pulsed with PHA. They were then incubated for 72 hours. Control cultures were handled in the identical fashion without pre-incubation with Ulex europeus seed extract. Harvesting and counting were performed as previously described for DNA synthesis.

Statistical Methods

Dixon's gap test

Any biological assay always includes several uncontrolled sources of variance influencing the final observation. In order to minimize this variation, assays are usually done in replicates from which arithmetic mean and variance are computed. However, such a sample may

have essentially a normal distribution except for one or more extreme observations at its upper, lower, or even both limits. If an extreme value actually differs only by chance, its rejection has less effect upon the mean estimated from the remaining sample than upon the variance. However, in either case the bias from rejecting a valid observation is usually far less than that caused by retaining a contaminant (98). Because these risks are so unequal, in the current experiments, all cultures were set up in five replicates and the Dixon's gap test (99) was used to reject any outlying values (two-tailed test) before computing their mean and variance.

Analysis of variance

It has been generally accepted that lymphocyte reactivity to mitogens seen in a single individual may demonstrate relatively large variability from day to day. In addition, variations seen in relation to other individuals may be due to differences existing prior to the experiment (100, 101). In order to separate this source of variability from treatment effects, i.e., the influence of Ulex europeus seed extracts, ulexine, etc., and experimental error, computational procedures for a single factor experiments having repeated measures were used (102). Multifactorial analysis was utilized to evaluate the interaction between PHA, Ulex europeus seed extract and saliva in the anti-H neutralization experiment. The computational procedures employed were those described by Winer for factorial experiments in which some of the interactions are confounded.

Analysis of regression

The dose-curve responses of crude Ulex europeus seed extract and dialyzed Ulex europeus seed extract upon lymphocyte stimulation were evaluated with the use of analysis of regression (103).

RESULTS

Original Observation

Evaluation of Ulex europeus seed extract in the purification of lymphocytes for culturing led to the original observation presented in Figure I. Peripheral blood lymphocytes from an individual of blood group O were stimulated with phytohemagglutinin while exposed to 1% (V/V) crude extract of Ulex europeus seed. Lymphocytes not exposed to PHA showed no differences in tritiated thymidine uptake, confirming the lack of mitogenicity of the extract. PHA stimulated cells, however, when exposed to the same Ulex extract were highly affected and their thymidine incorporation was considerably reduced ($p < .01$). Since this was a new observation, experiments were designed to investigate the possibility that Ulex europeus seed extract was a potent inhibitor of lymphocyte metabolism.

Ulex europeus as an inhibitor of PHA-stimulation

To confirm the original observation, peripheral blood lymphocytes obtained from eight normal individuals were cultured in a similar fashion. In order to minimize all possible sources of uncontrolled variability in the test system, care was taken to choose individuals of the blood group O since Ulex europeus seed extract has a specific activity for H substance. No attempt was made to categorize this group further. Results of these experiments are presented in Table I. In each experiment, treated cultures were simultaneously pulsed with

Figure I

This figure shows the effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of unstimulated and PHA-stimulated peripheral blood lymphocytes. The results are expressed as the average CPM ± 1 standard deviation of five replicated cultures.

Figure I

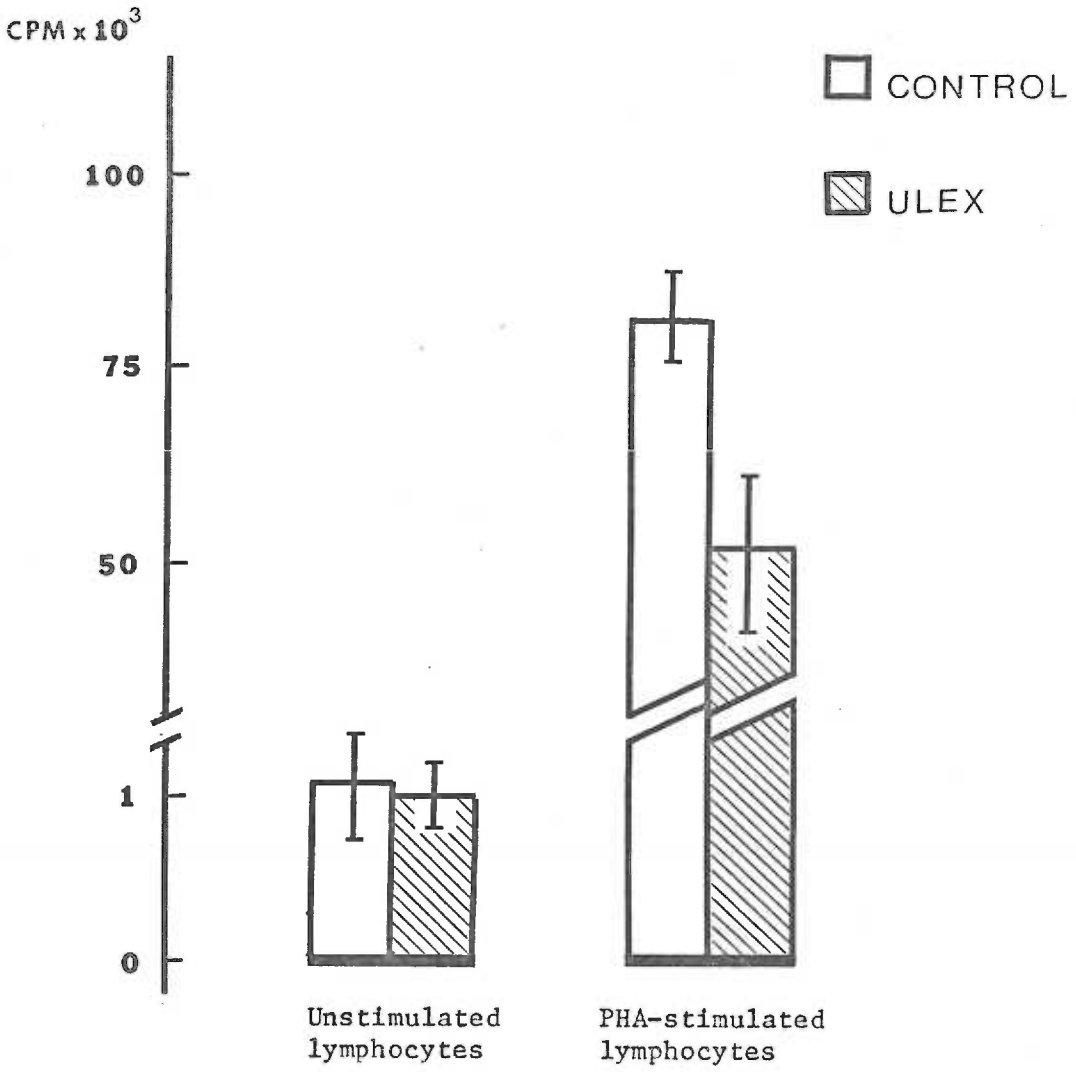

CPM x 10^3
100
75
50
1
0
CONTROL
ULEX
Unstimulated lymphocytes
PHA-stimulated lymphocytes

Table I

This table gives the data showing the effects of Ulex europeus seed extract [1% (V/V] upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes from individuals of blood group "O".

(Mean CPM ± 1 S.D.)

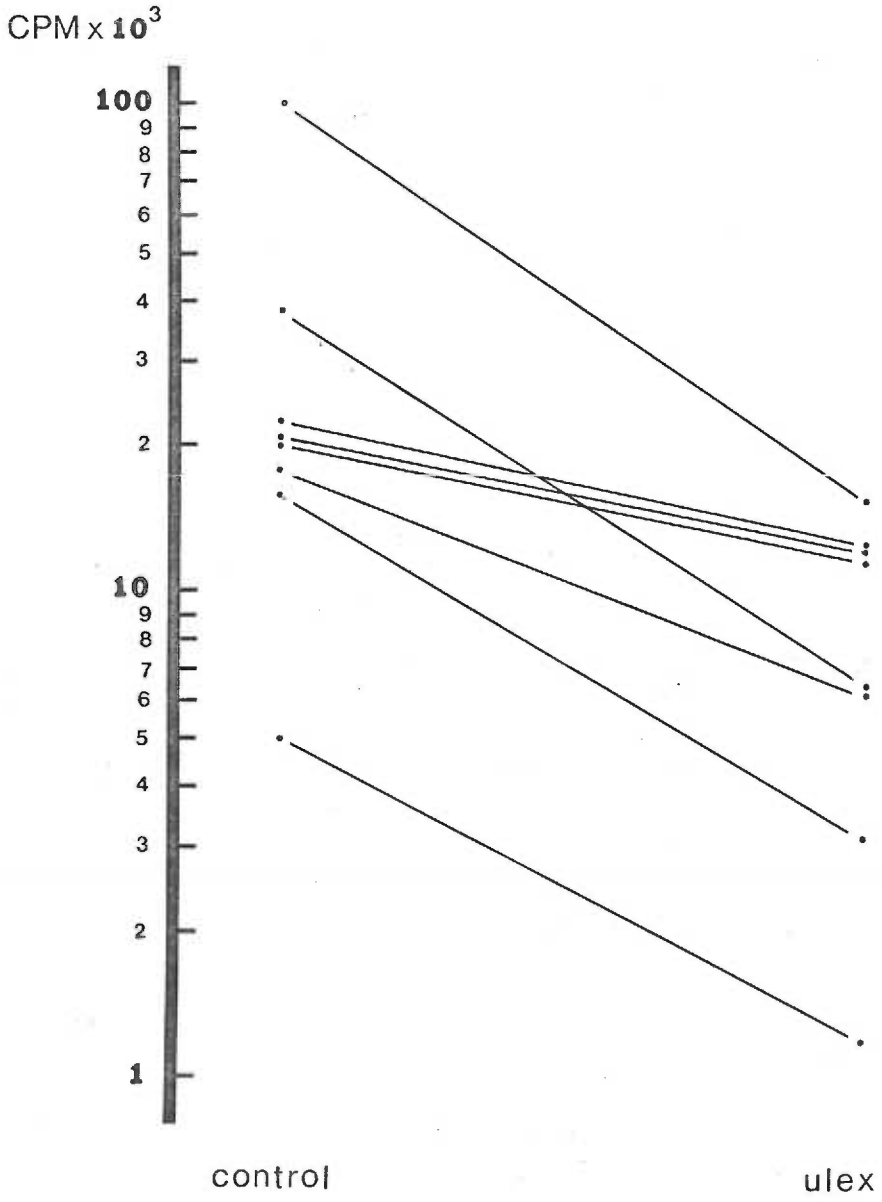
Table I

Person	Control	Ulex europeus extract 1% (V/V)	p
1	37575 ± 1400	6526 ± 610	<.01
2	20784 ± 4229	12727 ± 820	<.01
3	22088 ± 5391	12898 ± 821	<.01
4	18206 ± 3896	6258 ± 714	<.01
5	19876 ± 6329	12094 ± 1497	<.05
6	109233 ± 8129	15889 ± 2463	<.01
7	5089 ± 1030	1229 ± 382	<.01
8	15824 ± 2501	3401 ± 586	<.01

Figure II

Effects of Ulex europeus seed extract upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes from eight individuals of blood group "O". Each point is the mean value of five cultures. Standard deviations have been omitted for the sake of clarity.

Figure II



PHA and buffered saline. Results are expressed as the mean of Counts Per minute, ± one standard deviation of five replicates. A statistically significant inhibition ($p < .05$) of lymphocyte reactivity to PHA, in the presence of Ulex europeus extract was seen in all. To eliminate bias, results were pooled and subjected to the analysis of variance. Figure II illustrates the results. Variability in lymphocyte reactivity to PHA is obvious. Attempts to compare the results of control cultures with the results of cultures exposed to extract of Ulex europeus seed would not reveal a statistically significant difference. The variability of lymphocytes from different sources in reactivity to PHA is not the concern of the present experiments. It was therefore possible to reduce the variation of the experimental error by separating the total variation in a different partition. Single-factor experiments having repeated measures fall in with this arrangement and the two groups become statistically different ($p < .05$). Appendix I shows the computational procedures for this type of statistical test. These results confirm the original observation that Ulex europeus seed extract at a final concentration of 1% (V/V) inhibits tritiated thymidine uptake of PHA-stimulated lymphocytes from individuals of blood group O. In order to verify if the inhibition was related to the ABO blood group, peripheral blood lymphocytes of individuals of blood group A_1 were assayed in the same manner. The results are given in Table II. Each experiment shows a marked inhibition of tritiated thymidine uptake when Ulex europeus seed extract is present in cultures. Lymphocyte transformation of individuals of blood group

Table II

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes from individuals of blood group "A_1". (Mean CPM ± 1 S.D.)

Table II

Person	Control	Ulex europeus extract 1% (V/V)	p
1	14786 ± 1400	2206 ± 721	<.01
2	103376 ± 11285	45980 ± 7079	<.01
3	87667 ± 10334	16936 ± 4444	<.01
4	27334 ± 2774	7784 ± 868	<.01
5	87709 ± 8829	48220 ± 7958	<.01
6	18859 ± 3029	3412 ± 694	<.01

Figure III

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of unstimulated and pokeweed-stimulated peripheral blood lymphocytes.

(Mean CPM ± 1 S.D.)

Figure III

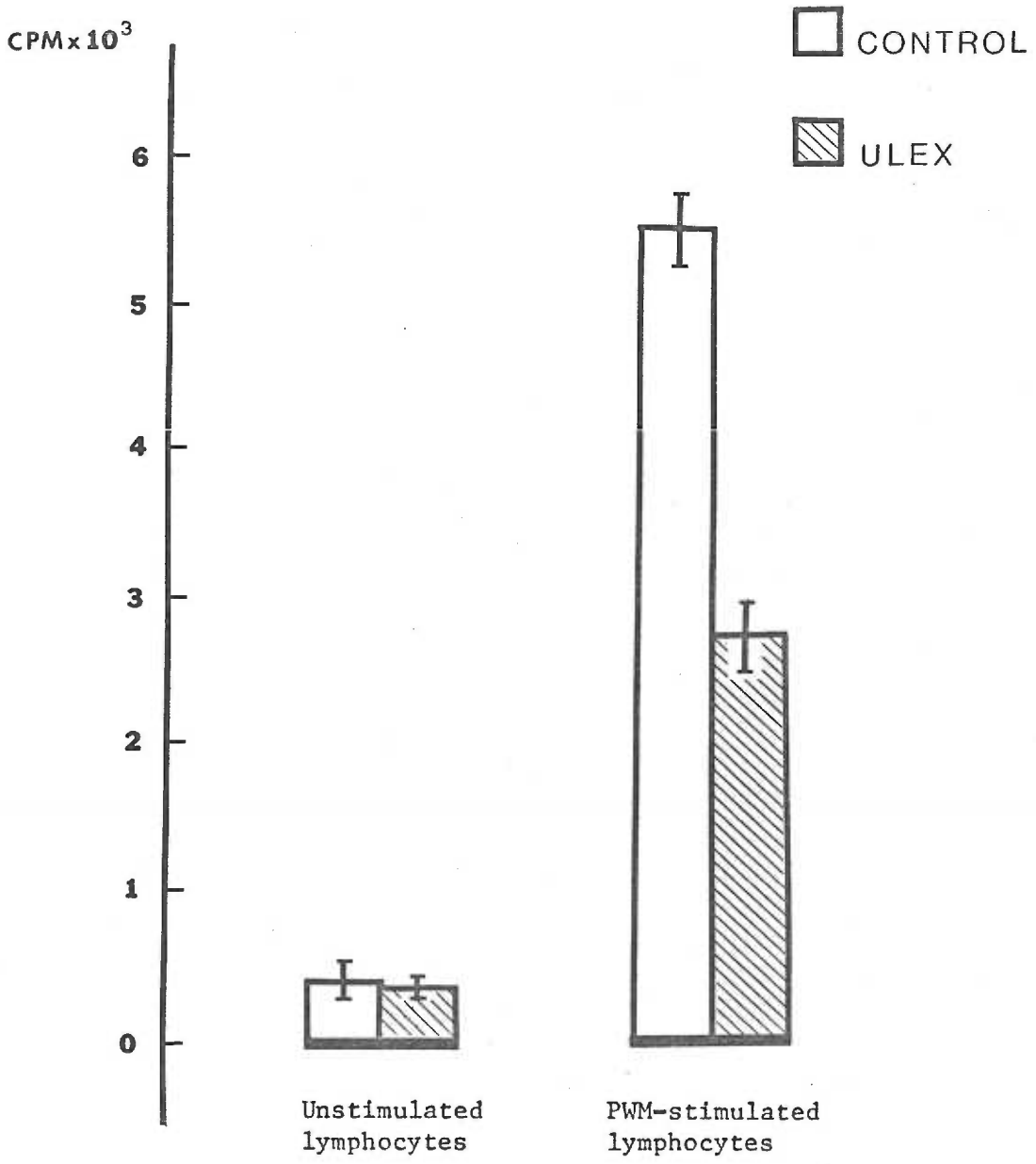
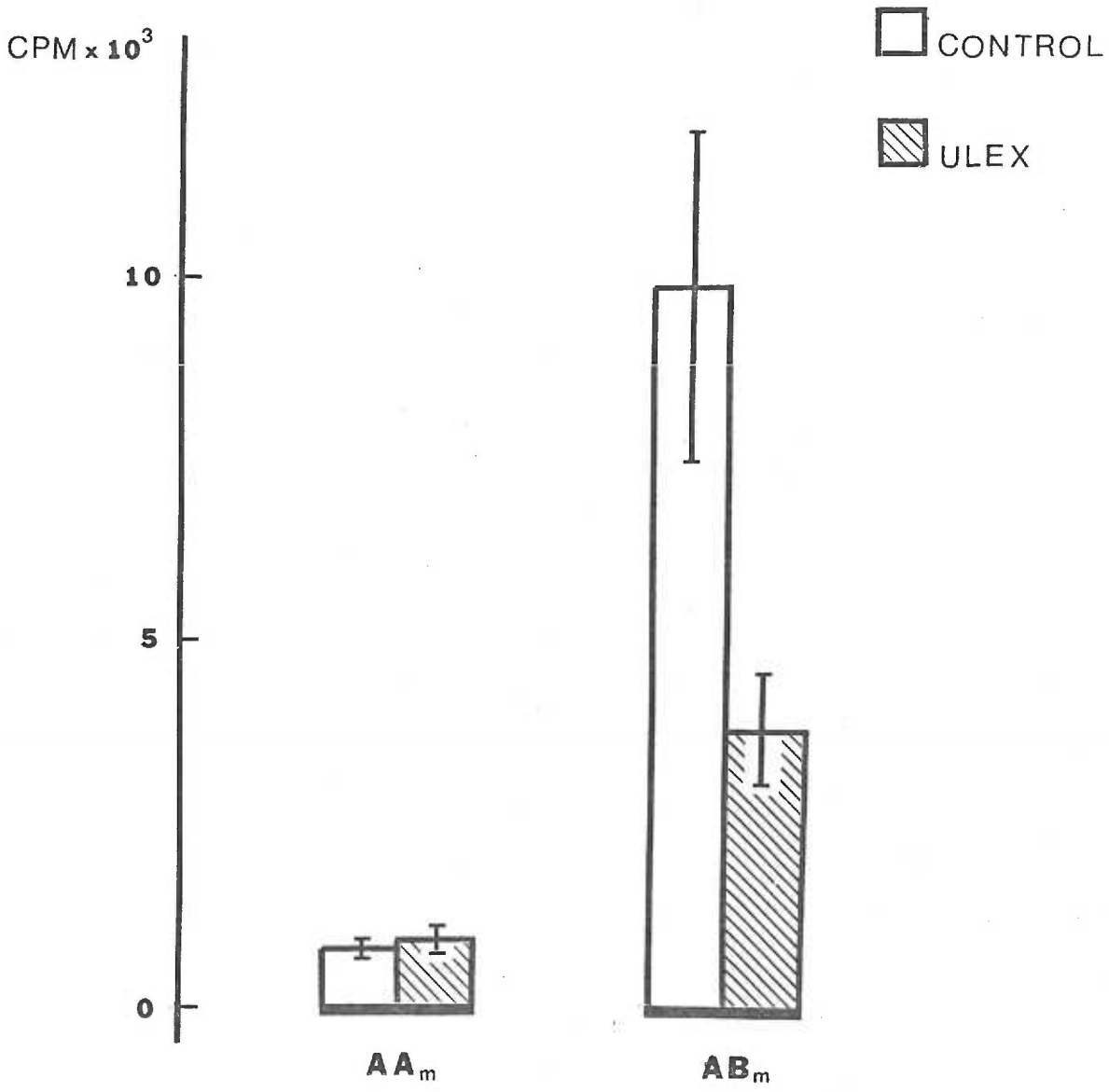

CPM x 10³
6
5
4
3
2
1
0
CONTROL
ULEX
Unstimulated lymphocytes
PWM-stimulated lymphocytes

Figure IV

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake in mixed lymphocyte reaction (MLR). AA_m represents the base-line values of reacting lymphocytes. AB_m represents a one-way mixed lymphocyte reaction where lymphocytes A are reacting cells and lymphocytes B are stimulating cells.
(Mean CPM ± 1 S.D.)

Figure IV



A_1 appears to be affected to the same extent as lymphocytes of individuals of blood group O. However, whether the amount of H substance varies in lymphocyte membranes according to the blood group as it does for the red cells, is not known. Nevertheless, further experiments were carried out with lymphocytes from individuals of blood group O.

Competitive inhibition as the possible mechanism of action of Ulex europeus

The mechanism whereby PHA induces blastogenesis and mitosis in normal thymus-dependent lymphocytes in tissue culture is still highly controversial in spite of the large number of studies which have been carried out over the past few years. In order to see if Ulex europeus seed extract was specifically inhibitory for PHA lymphocyte transformation, experiments were carried out with pokeweed mitogen (PWM). Results are presented in Figure III. The same inhibition was observed. Ulex europeus seed extract had no affect on unstimulated lymphocytes but significantly inhibited tritiated thymidine uptake of PWM-stimulated lymphocytes ($p < .01$).

The possibility of a competitive mechanism between the various plant lectins was investigated. The influence of Ulex europeus seed extract seed on mixed lymphocyte cultures (MLC) was studied. Figure IV illustrates the pattern of tritiated thymidine uptake at the end of five days of incubation in a typical mixed lymphocyte reaction system. Control cultures (AA_m) did not exhibit inhibition of tritiated thymidine uptake after exposure to Ulex europeus seed extract. In contrast, a marked inhibition was noted in mixed lymphocyte

Figure V

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of unstimulated peripheral blood lymphocytes. Each point represents the mean value of five replicated cultures. Standard deviations have been omitted for the sake of clarity.

Figure V

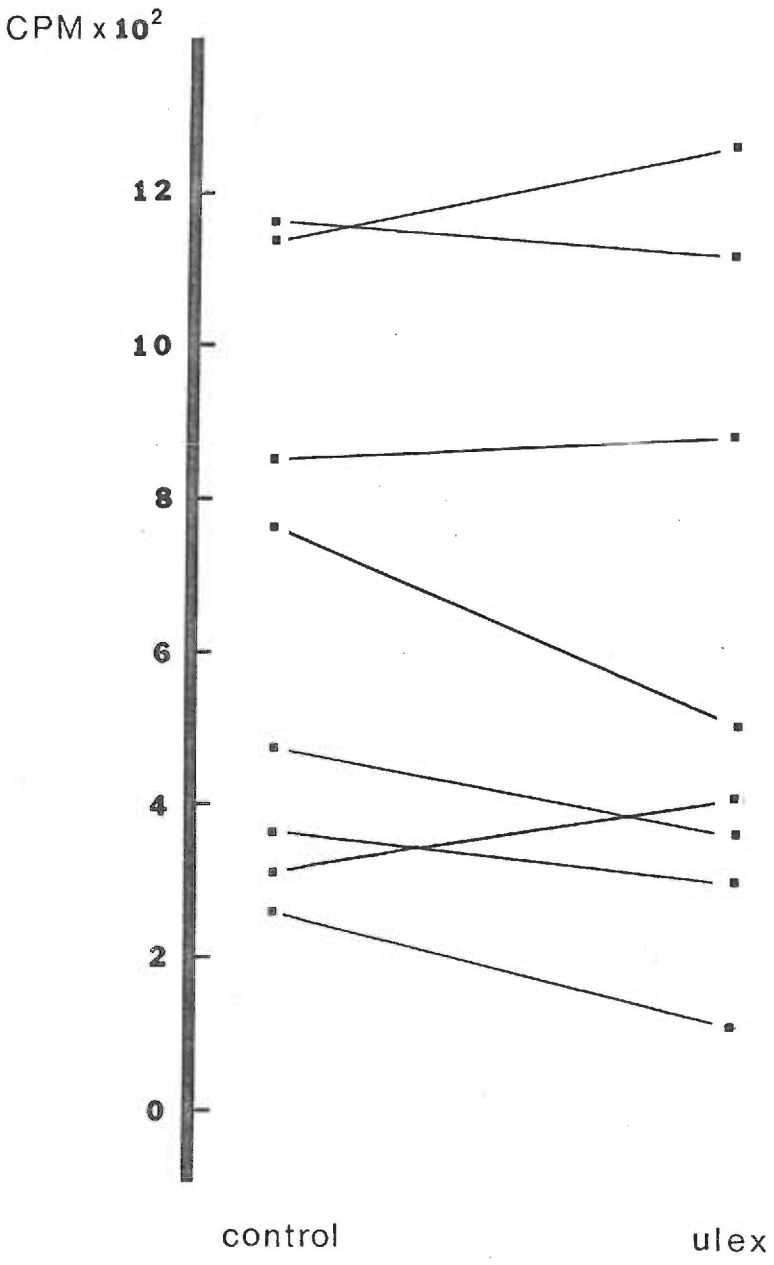
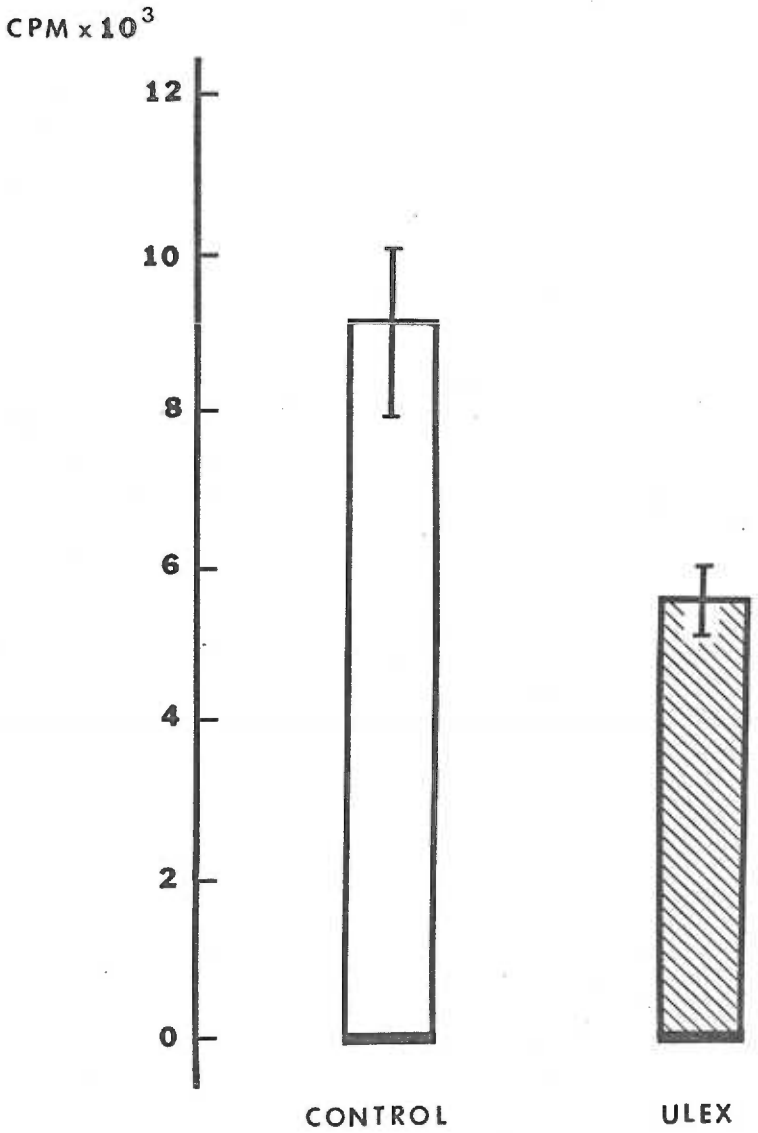


Figure VI

Effects of Ulex europeus seed extract [1% (V/V)] upon the spontaneous tritiated thymidine uptake of lymphoid cells from the Itwazuki cell line, after three days of incubation. (Mean CPM ± 1 S.D.)


CPM x 10³
12
10
8
6
4
2
0
CONTROL
ULEX

reaction (AB_m) ($p < .01$). These results suggest that lymphocyte replication is inhibited as well as lymphocyte differentiation. In addition, the interference of Ulex in mixed lymphocyte reactions makes it unlikely that the mechanism of action in PHA and PWM lymphocyte transformation experiments is dependent on competitive inhibition.

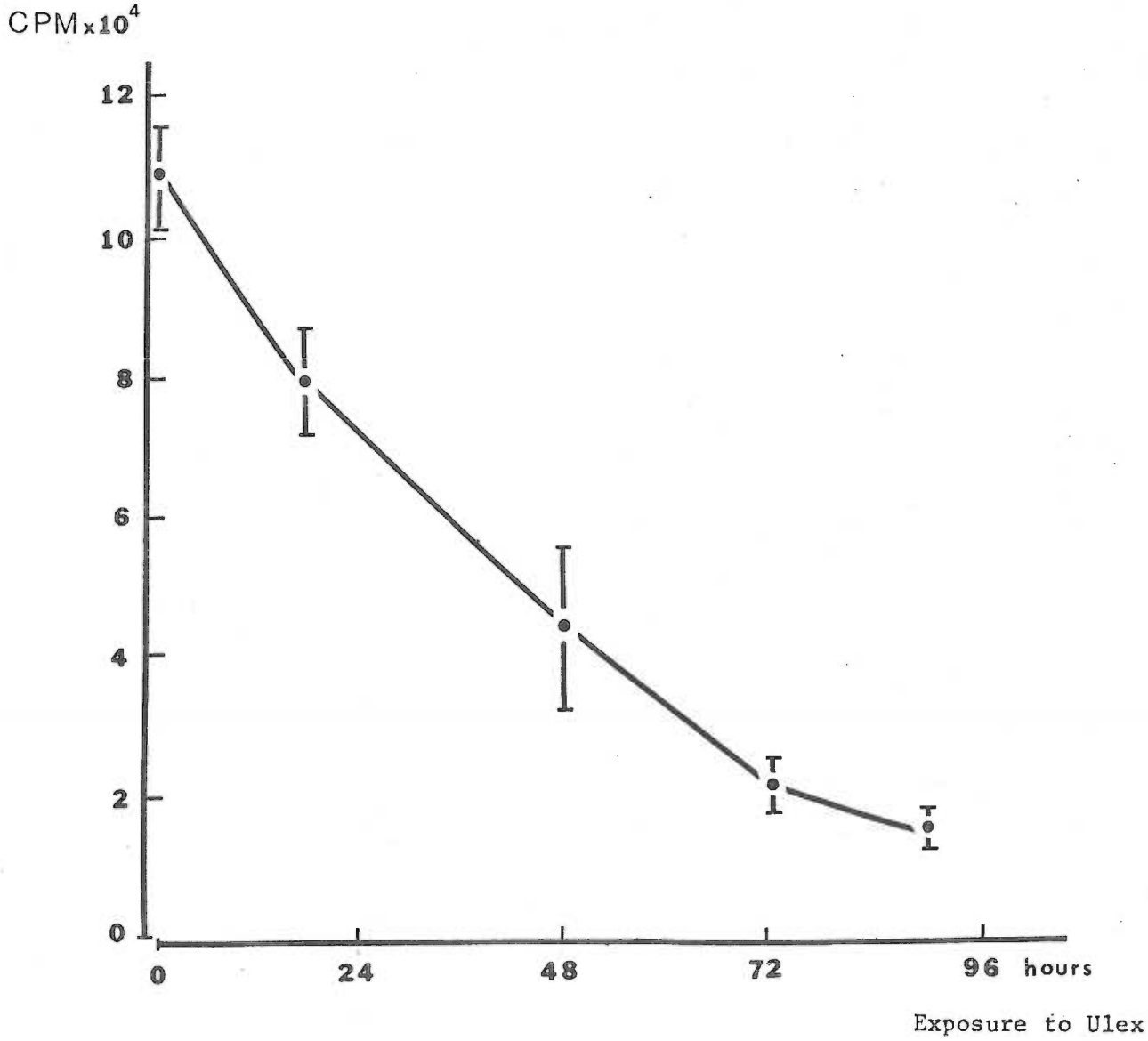
Is activation process necessary for the inhibitory effect of Ulex europeus?

Ulex europeus seed extract appeared inhibitory irrespective of the stimulatory source. Similar inhibitory findings were obtained with plant mitogens or alloantigens. The importance of lymphocyte activation upon the inhibitory capacity of Ulex europeus seed extract was studied. A lymphocyte culture test system was established without mitogen. Cultures were initiated as with lymphocyte transformation experiments. Results are presented in Figure V. Statistical analysis to eliminate individual variability was performed. A significant difference was not obtained. Variability within each set of replicates overwhelmed any possible effect of Ulex europeus seed extract. In order to eliminate this difficulty, lymphoid cells with high spontaneous rate of DNA synthesis had to be utilized. Such features are observed in lymphoid cells maintained in permanent cultures. In the following experiments, lymphoid cells from Itwazuki cell line were used. Results are shown in Figure VI. Cells incubated with Ulex europeus seed extract for a period of three days had a marked inhibition of DNA synthesis as reflected by a lower incorporation of tritiated

Figure VII

Effects of length of exposure to Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

Figure VII



thymidine ($p < .01$).

Influence of length of exposure to Ulex europeus seed extract upon the lymphocyte thymidine uptake

The influence of length of exposure to Ulex europeus seed extract upon PHA-stimulated lymphocytes was studied in the following experiment: A set of five replicated cultures containing peripheral blood lymphocytes with PHA were pulsed with Ulex europeus seed extract at the start of incubation. A new set was then pulsed at 24-hour intervals up to 72 hours from the start of the study. A set of control cultures were maintained unexposed to the extract. All cultures were harvested at the same time. Accordingly, every culture had an equal length of exposure to PHA, but an unequal length of exposure to Ulex europeus extract. The results of this experiment are illustrated in Figure VII. The degree of inhibition induced by Ulex europeus seed extract is directly related to the length of exposure of the PHA-stimulated cells to the extract. The greater the exposure time of stimulated cells to Ulex europeus extract, the more reduced was the thymidine incorporation. It is of interest to note that incubation of lymphocytes with the plant extract for a period as short as 18 hours is sufficient to induce a marked inhibition in the thymidine uptake ($p < .01$).

Effect of Ulex europeus seed extract upon lymphocyte-T_o

Prior studies have established the reproducibility and accuracy

Figure VIII

Effects of length of incubation upon the coefficient of variation of unstimulated peripheral blood lymphocytes.

($r = .997$)

Figure VIII

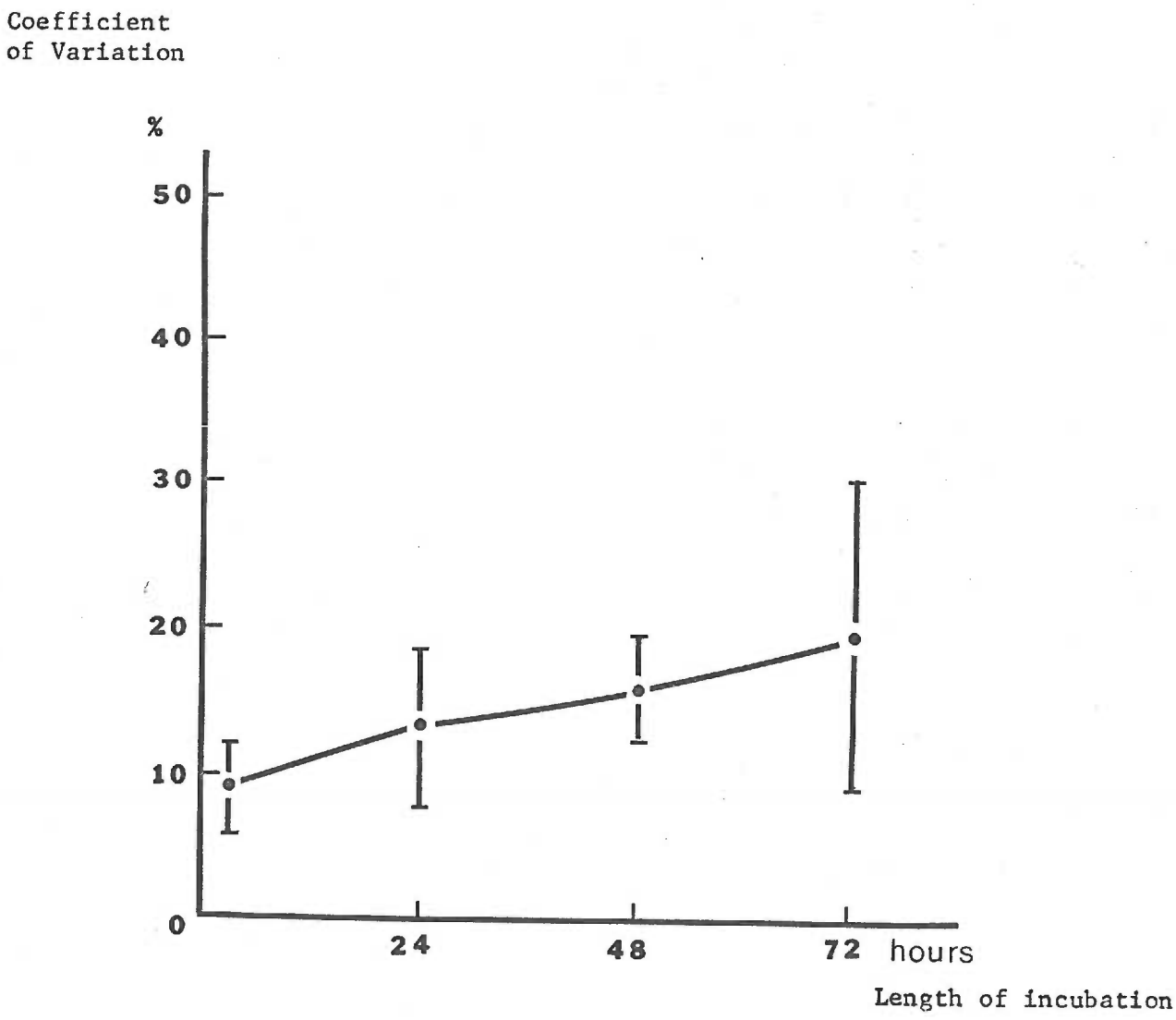

Coefficient
of Variation
%
50
40
30
20
10
0
24
48
72
hours
Length of incubation

Table III

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of peripheral blood lymphocytes in T_o-tests. (Mean CPM ± 1 S.D.)

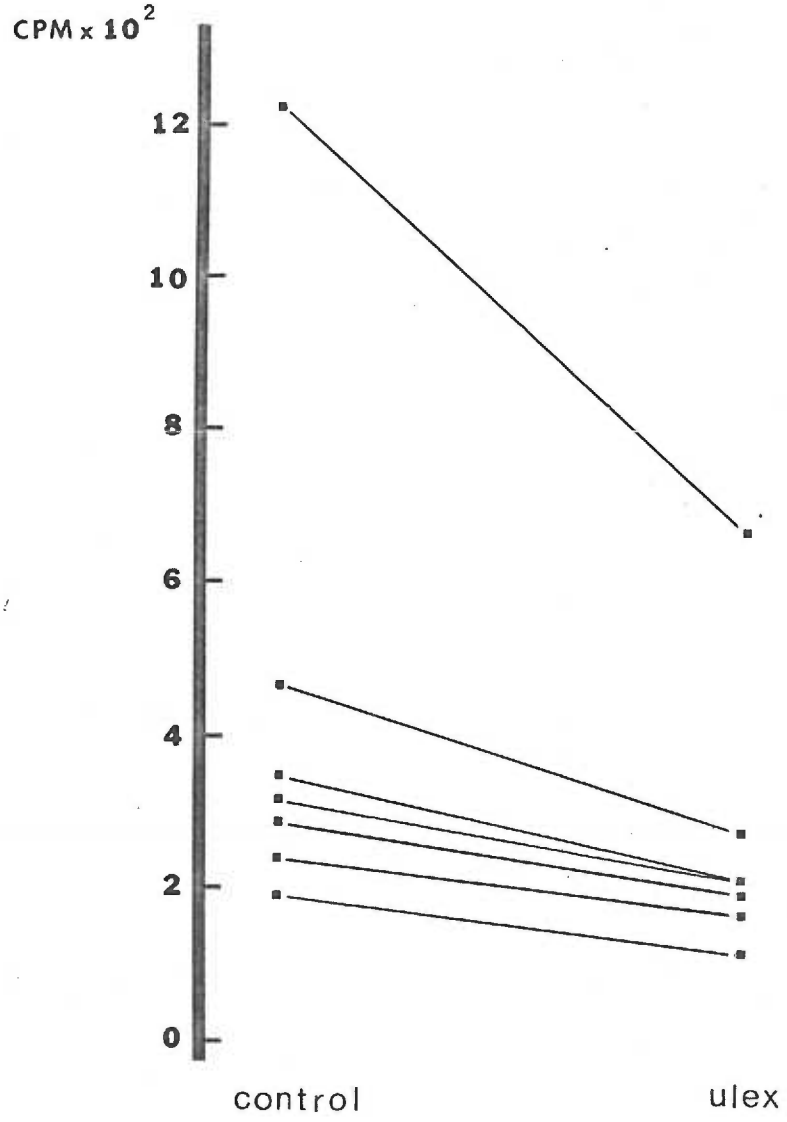
Table III

Person	Control	Ulex europeus extract 1% (V/V)	p
1	240 ± 25	171 ± 13	<.01
2	1224 ± 68	674 ± 34	<.01
3	462 ± 73	275 ± 29	<.01
4	284 ± 43	198 ± 20	<.01
5	316 ± 35	211 ± 25	<.01
6	344 ± 22	204 ± 17	<.01
7	196 ± 28	126 ± 12	<.01

Figure IX

Effects of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of peripheral blood lymphocytes in T_o-tests. Each point represents the mean value of five replicated cultures. Standard deviations have been omitted for the sake of clarity.

Figure IX



of the semi-micro technique of lymphocyte culture used in the present experiments. It was determined that the coefficient of variation, in part, was directly related to the length of incubation. Results from 20 sets of five replicated determinations were examined to provide a quantitative description of variability among these determinations. A variance estimate was calculated for each set of replicates and was averaged overall for the 20 sets at different intervals. It was noted that the shorter the incubation with unstimulated cells, the smaller the variability among the replicates (Figure VIII). In order to take advantage of the rapid action of Ulex europeus seed extract and the reduced variability in short experiments, T_o-tests on peripheral blood lymphocytes were performed. This test mirrors the actual rate of DNA synthesis of an individual's lymphocytes and the degree of thymidine incorporation will vary according to the individual's immune responsiveness at that specific time. Each experiment was analyzed separately and results are presented in Table III. In addition, data were pooled and studied with the use of the analysis of variance as previously described in Appendix I. The results are summarized in Figure IX. It was observed that Ulex europeus seed extract significantly reduced the spontaneous thymidine uptake of peripheral blood lymphocytes ($p < .01$). A three-hour contact with lymphocytes was sufficient to induce a significant change in DNA metabolism.

Toxicity studies

It is evident that the above observation could be the result of a

Figure X

Effects of Ulex europeus seed extract [1% (V/V)], mitomycin C (40 µg/ml) and heat (45°C for 30 min) upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

Figure X

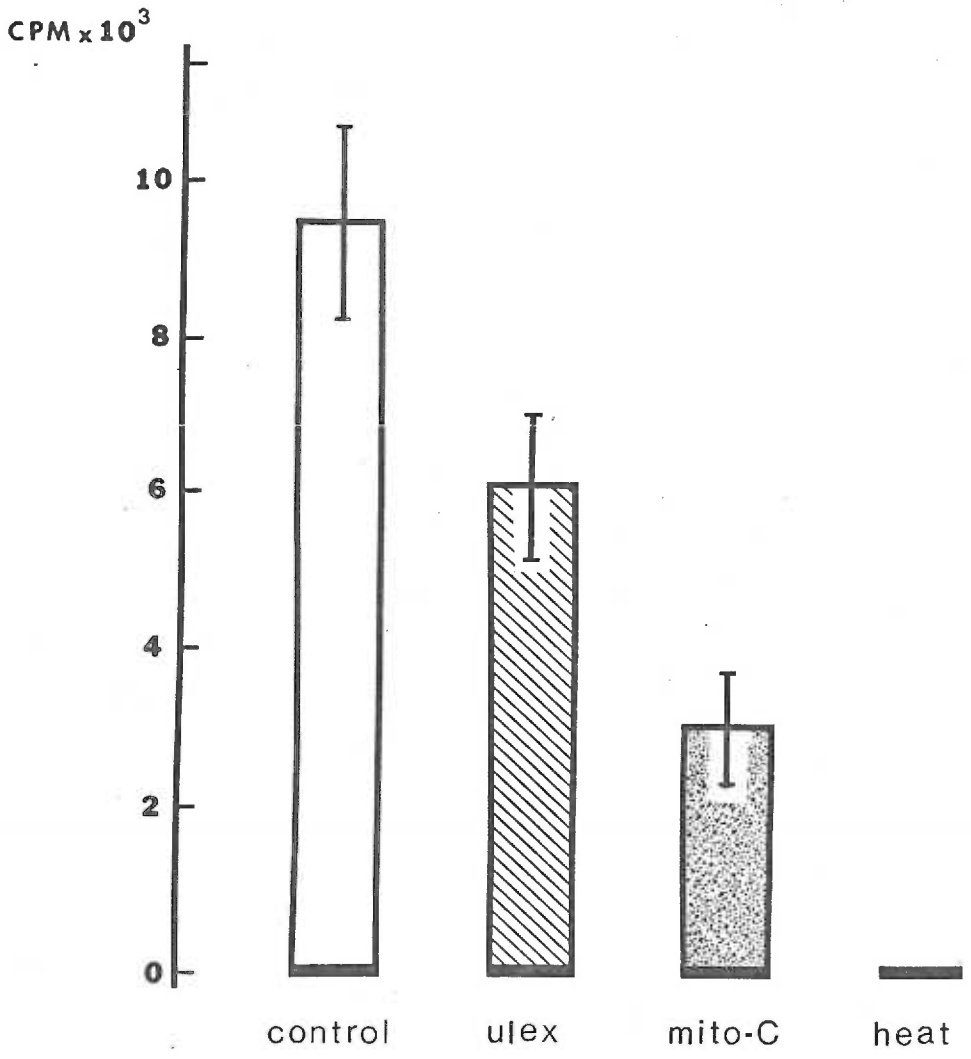


Table IV

Effects of Ulex europeus seed extract at a concentration of 1 and 5% (V/V) upon the viability of unstimulated peripheral blood lymphocytes. Results are expressed as percentage of viable cells as evaluated by trypan blue exclusion. (Mean % ± 1 S.D. of triplicate cultures)

Table IV

Length of incubation (hours)	Control % Viable cells	Ulex eur. 1% (V/V) % Viable cells	Ulex eur. 5% (V/V) % Viable cells
20	97 ± 1	97 ± 1	97 ± 1
44	87 ± 4	85 ± 5	82 ± 5
68	68 ± 6	72 ± 5	71 ± 6
92	50 ± 10	48 ± 9	54 ± 12

toxic action of Ulex europeus seed extract. Alternatively, it may have a selective anti-DNA activity such as mitomycin. Figure X illustrates the results of an experiment where PHA-stimulated peripheral blood lymphocytes were treated in three distinct systems. One set of cultures were incubated with Ulex europeus seed extract. A second set contained mitomycin-treated cells; a third set of cultures were heated at 45°C for 30 min. In all three sets of lymphocyte cultures, there was a significant inhibition of the tritiated thymidine uptake ($p < .01$).

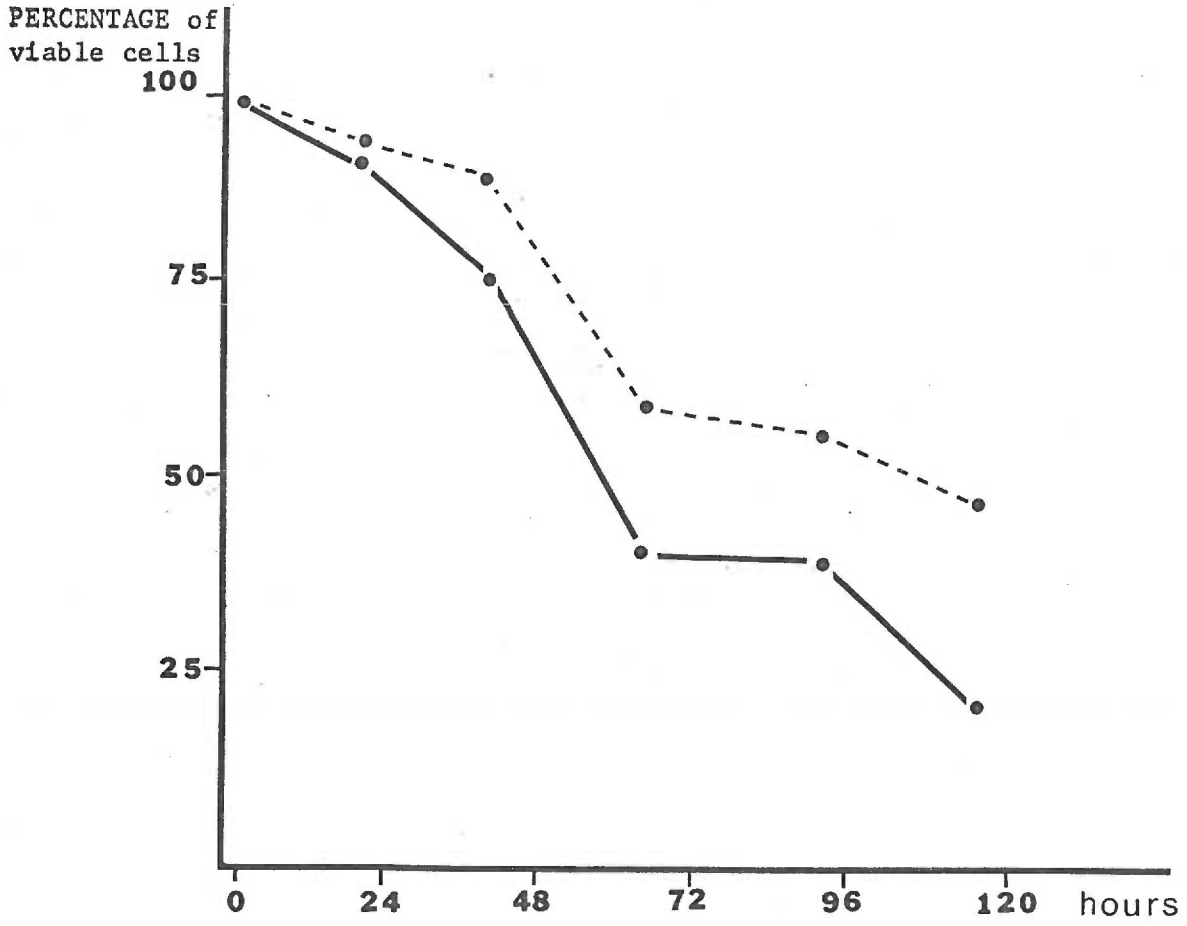
In order to determine if the observed inhibition was the result of a cytotoxic effect of Ulex europeus seed extract, three types of experiments were designed. The first group employed dye exclusion ability. Dead cells cannot exclude vital dyes. Accordingly, the effect of Ulex europeus seed upon lymphocyte viability was studied by dye exclusion. Major damage to the lymphocyte membrane would be detected by staining of the cells. The residual low tritiated thymidine uptake would be explained by the remaining viable lymphocytes.

Unstimulated peripheral blood lymphocytes were incubated in the presence of 1 and 5% (V/V) crude extract of Ulex europeus seeds for a period up to 92 hours. Cultured cells were sequentially studied for viability with trypan blue staining. Results are presented in Table IV. The data indicate that numbers of viable cells do not differ significantly between control cultures and cultures containing Ulex europeus seed extract. Peripheral blood lymphocytes are known to be resistant to damaging conditions and have an excellent survival in vitro. Accordingly, the studies were expanded to include human

Figure XI

Viability of human thymocytes evaluated with the use of trypan blue. ●——● represents control cultures. ●----● represents cultures containing 5% (V/V) of Ulex europeus seed extract.

Figure XI


PERCENTAGE of viable cells
100
75
50
25
0
24
48
72
96
120
hours
Length of incubation

thymocyte viability. These cells show a poor survival capacity in vitro and are generally more sensitive than peripheral blood lymphocytes to any deleterious conditions. Thymocyte cultures were exposed to Ulex europeus seed extract at a final concentration of 5% (V/V). Viability evaluation was performed sequentially. The results are presented in Figure XI. The data suggest that Ulex europeus seed extract is not cytotoxic and actually appears to prolong human thymocyte survival in vitro. Further studies are required to confirm this impression. However, the dye exclusion procedure has shown that Ulex europeus seed extract at a final concentration of 1 and 5% (V/V) does not cause major damage to the lymphocyte membrane.

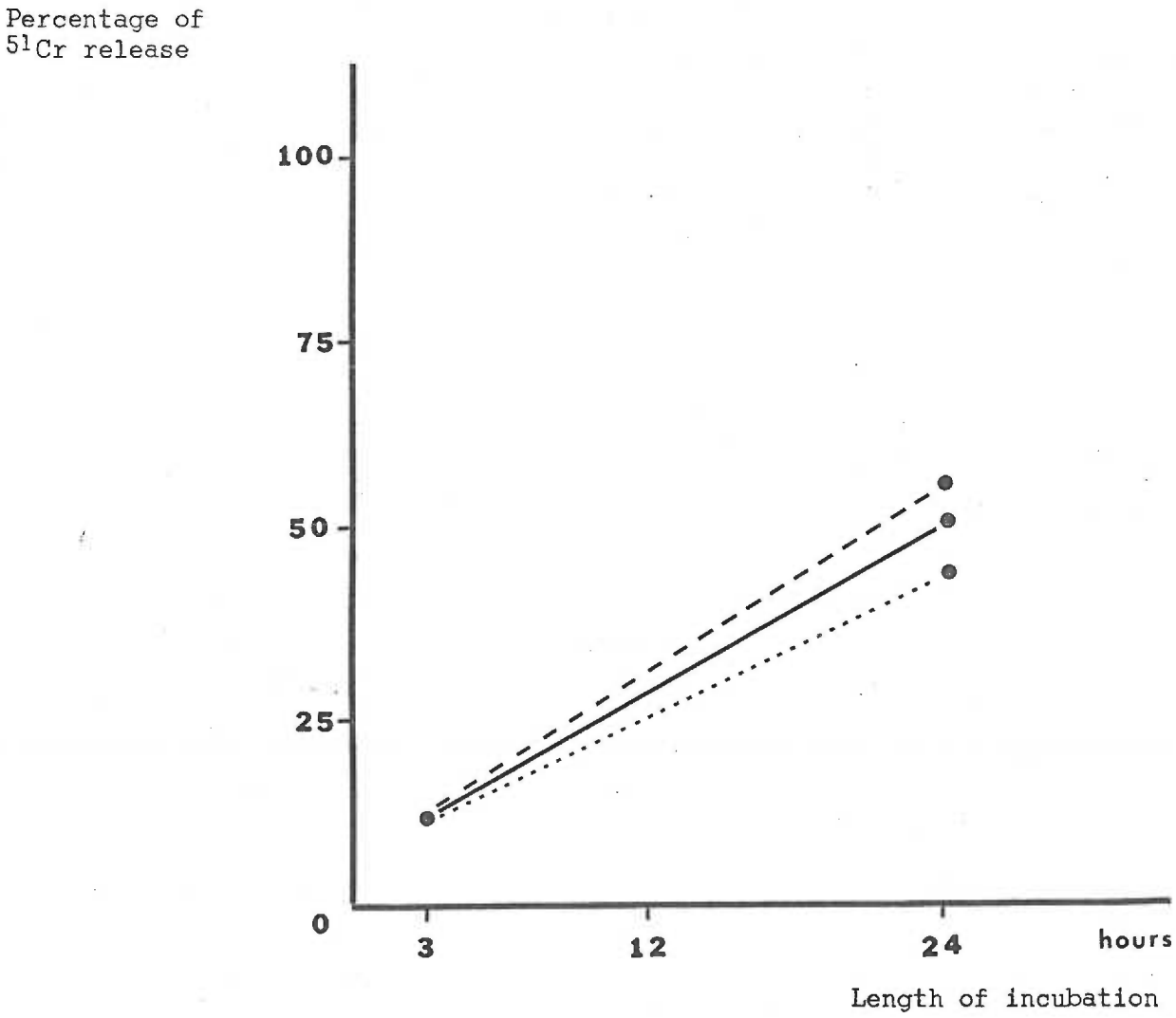
Unfortunately, dye exclusion techniques are generally insensitive and may only demonstrate major membrane damage. Minimal damage to the cell membrane may be overlooked; a more sensitive assay was needed to arrive at a definitive conclusion.

Radioactive isotope release is frequently employed to evaluate cytotoxicity. Although it is more sensitive than the dye exclusion technique, it has certain disadvantages such as a spontaneous release of the isotope over a period of time. The nonspecific release of isotope greatly limits the period during which viability assays can be performed. From the previous experiments, it was shown that a three-hour incubation was sufficient to observe the inhibition of the tritiated thymidine uptake secondary to exposure to Ulex europeus seed extract. Peripheral blood lymphocytes were labeled with ^{51}Cr and were incubated with a 1 and 5% (V/V) concentration of Ulex europeus seed

Figure XII

Effects of Ulex europeus seed extract upon ^{51}Cr release from labeled peripheral blood lymphocytes. ●——● represents the controls, ●----● Ulex europeus seed extract 1% (V/V), and ●····● Ulex europeus seed extract 5% (V/V).

Figure XII


Percentage of
51Cr release
100
75
50
25
0
3
12
24
hours
Length of incubation

extract for 3 and 24 hours respectively. Chromium release in the supernatant was counted and compared to the control. Figure XII illustrates the results. After a three-hour incubation, there is no difference in the amount of chromium release between control supernatants and supernatants from cultures containing 1 and 5% of Ulex europeus seed extract. After 24 hours of incubation, there is still no difference between the controls and cultures exposed to 1% concentration of the extract. However, supernates from cultures containing 5% concentration of the plant extract were less radioactive than the controls ($p < .02$). This finding is similar to that found with the dye exclusion procedures. It suggests that a concentration of 5% (V/V), Ulex europeus seed extract may protect the integrity of the lymphocyte membrane.

There are other mechanisms leading to a reduction of tritiated thymidine; as shown in Figure X, cells can be treated with drugs such as mitomycin C or irradiated. They will remain viable as determined by both dye exclusion and chromium release. Nevertheless, such treated cells cannot synthesize new DNA and replication is virtually impossible. Ulex europeus seed extract could thus affect lymphocytes in a similar fashion by impairing their DNA synthesizing apparatus. In an attempt to overcome this objection and reinforce the impression of a lack of cytotoxicity, further experiments were designed. An attempt was made to determine if the observed inhibition was permanent or reversible. Peripheral blood lymphocytes were incubated with Ulex europeus seed extract for a 48 and 72-hour period. They were then freed of the

Figure XIII

Effects of pre-incubation with Ulex europeus seed extract at a concentration of 1% and 5% (V/V) upon the reactivity of peripheral blood lymphocytes to PHA. Cells were washed free of extract prior to addition of PHA. (Mean CPM ± 1 S.D.)

Figure XIII

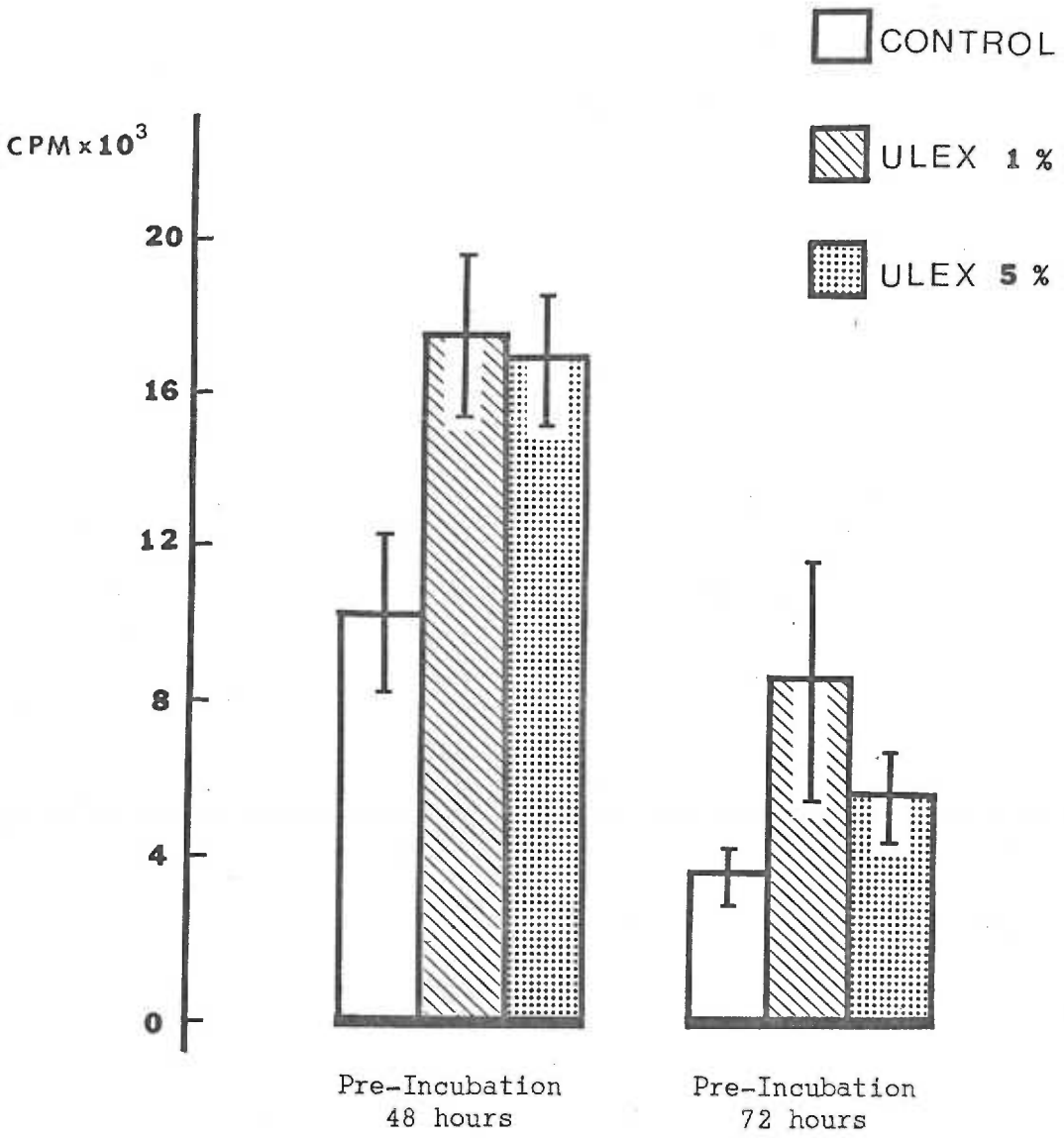
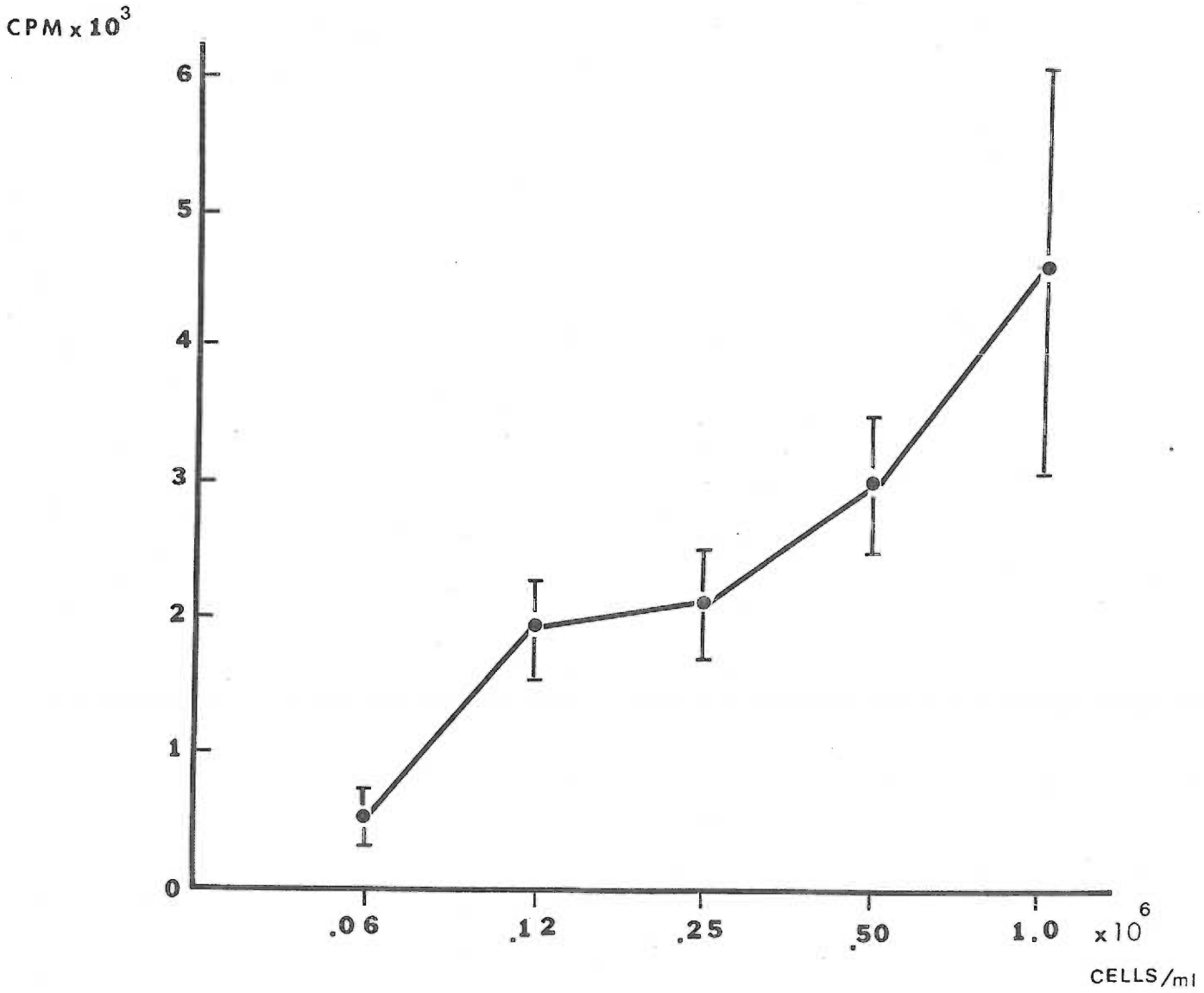

CONTROL
ULEX 1 %
ULEX 5 %
CPM x 10³
20
16
12
8
4
0
Pre-Incubation
48 hours
Pre-Incubation
72 hours

Figure XIV

Effect of the number of lymphocytes on the incorporation of tritiated thymidine in semi-micro PHA transformation assay. Each point represents the mean CPM ± 1 S.D. of five parallel cultures.

Figure XIV



extract by washings and stimulated with PHA. Results are presented in Figure XIII. It was found that lymphocytes which were exposed to the plant extract respond to phytohemagglutinin as well as the control cells, if not better. Indeed, treated cells showed an increase of their thymidine uptake over the control cells ($p < .05$). In order to evaluate this impression, further experiments were carried out to determine the cell density that would give optimum levels of thymidine incorporation in the present lymphocyte culture technique. It is known that too many cells in a culture lead to minimal responses to PHA, while a smaller number react optimally. Results presented in Figure XIII could be explained on this basis and toxicity of Ulex europeus seed extract would be overlooked. Using the semi-micro lymphocyte culture technique, five densities of lymphocytes were assayed. Cell densities used were 1.0, 0.5, 0.25, 0.12 and 0.06 X 10^6 cells/ml of tissue culture medium. Figure XIV illustrates the results. It was observed that within the range of cell concentration used, lymphocyte tritiated thymidine uptake was proportional to the number of lymphocytes present in the culture tube ($r = 0.953$). Therefore, results presented in Figure XIII cannot be explained on the basis of fewer reacting lymphocytes, in contrast to control cultures, after exposure to Ulex europeus seed extract. On the contrary, the data suggest that there were more remaining viable cells capable of reacting to PHA. This observation supports the findings of the previous experiments, that Ulex europeus seed extract is non-toxic for lymphocytes.

Figure XV

Effect of Ulex europeus seed extract [1% (V/V)] upon ^{14}C-leucine incorporation of unstimulated and PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

Figure XV

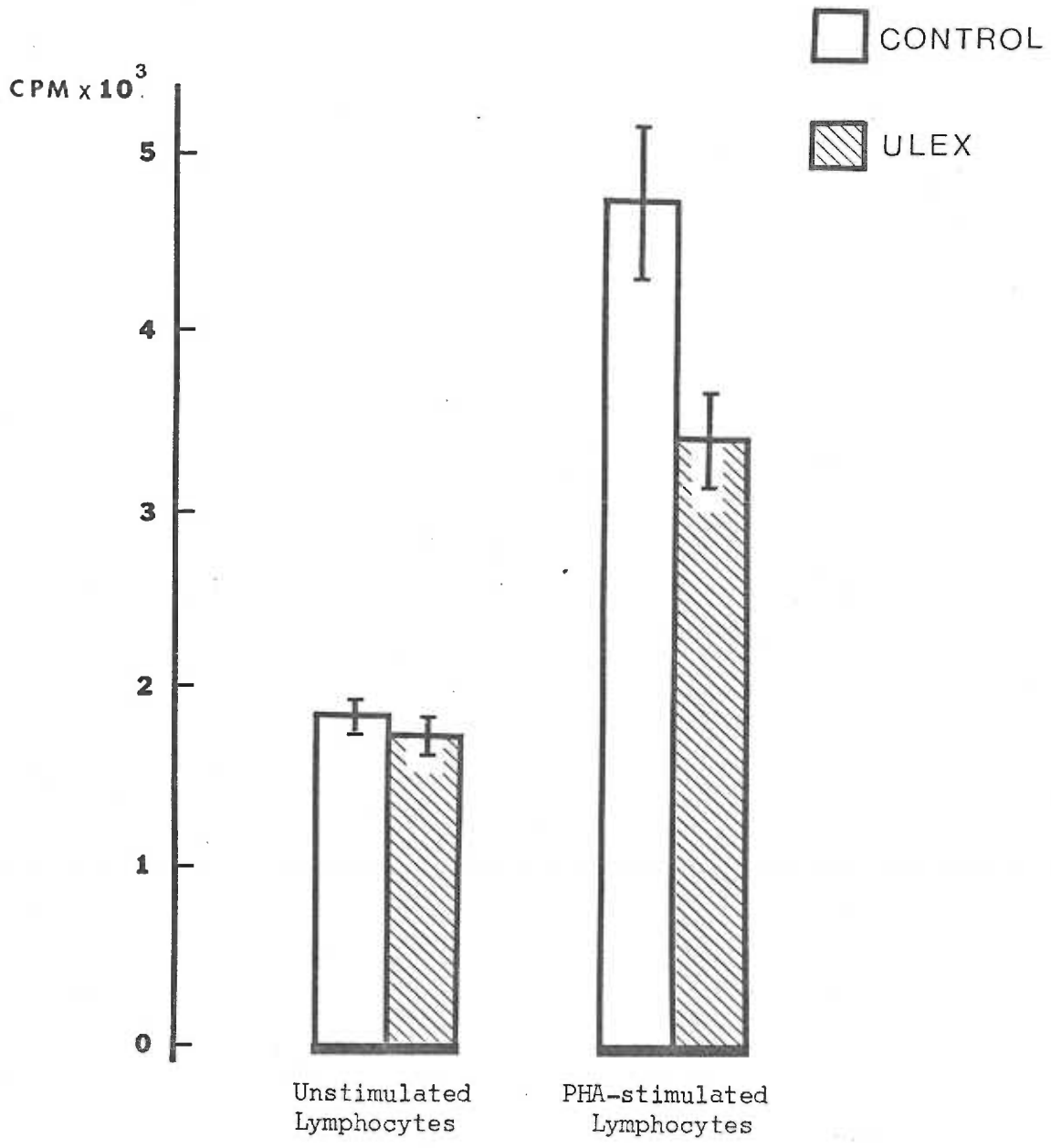
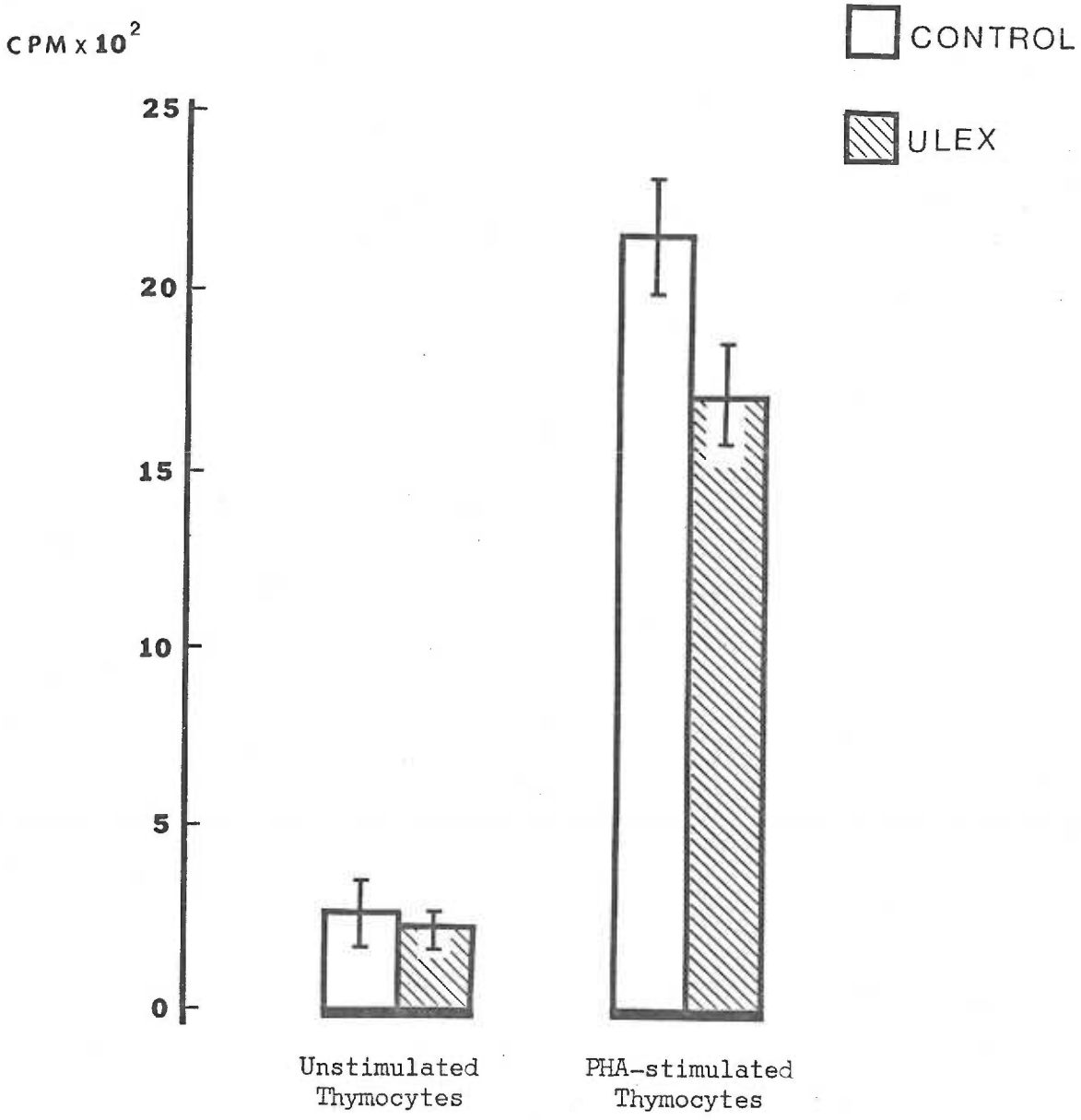

CONTROL
ULEX
CPM x 10³
5
4
3
2
1
0
Unstimulated Lymphocytes
PHA-stimulated Lymphocytes

Figure XVI

Effect of Ulex europeus seed extract [1% (V/V)] upon tritiated thymidine uptake of unstimulated and PHA-stimulated human thymocytes. (Mean CPM ± 1 S.D.)

Figure XVI


CPM x 10²
CONTROL
ULEX
25
20
15
10
5
0
Unstimulated Thymocytes
PHA-stimulated Thymocytes

Effect of Ulex europeus seed extract upon lymphocyte protein synthesis

In order to evaluate the effect of Ulex europeus seed extract upon lymphocyte protein synthesis, PHA-transformation experiments were performed. Such activated cells supply one of the best models for metabolic studies. Results presented in Figure XV indicate that protein synthesis of PHA-stimulated lymphocytes is inhibited in the presence of Ulex europeus seed extract. The ^{14}C-leucine incorporation is significantly reduced when compared to the controls ($p < .01$). Significant differences were not found when unstimulated lymphocytes were studied.

Effect of Ulex europeus seed extract upon ^{3}H-thymidine uptake of lymphocytes from different sources

The following experiments were designed to determine if Ulex europeus seed extract had selective inhibitory effects upon lymphocytes obtained from several sources. Most of the previous experiments were performed with peripheral blood lymphocytes. These cells are considered to be a mixture of thymus-derived lymphocytes or T-cells and thymus-independent or B-cells; T-type lymphocytes are most numerous in the peripheral circulation. Attempts were made to selectively evaluate the effect of Ulex europeus seed extract upon T and B cells respectively.

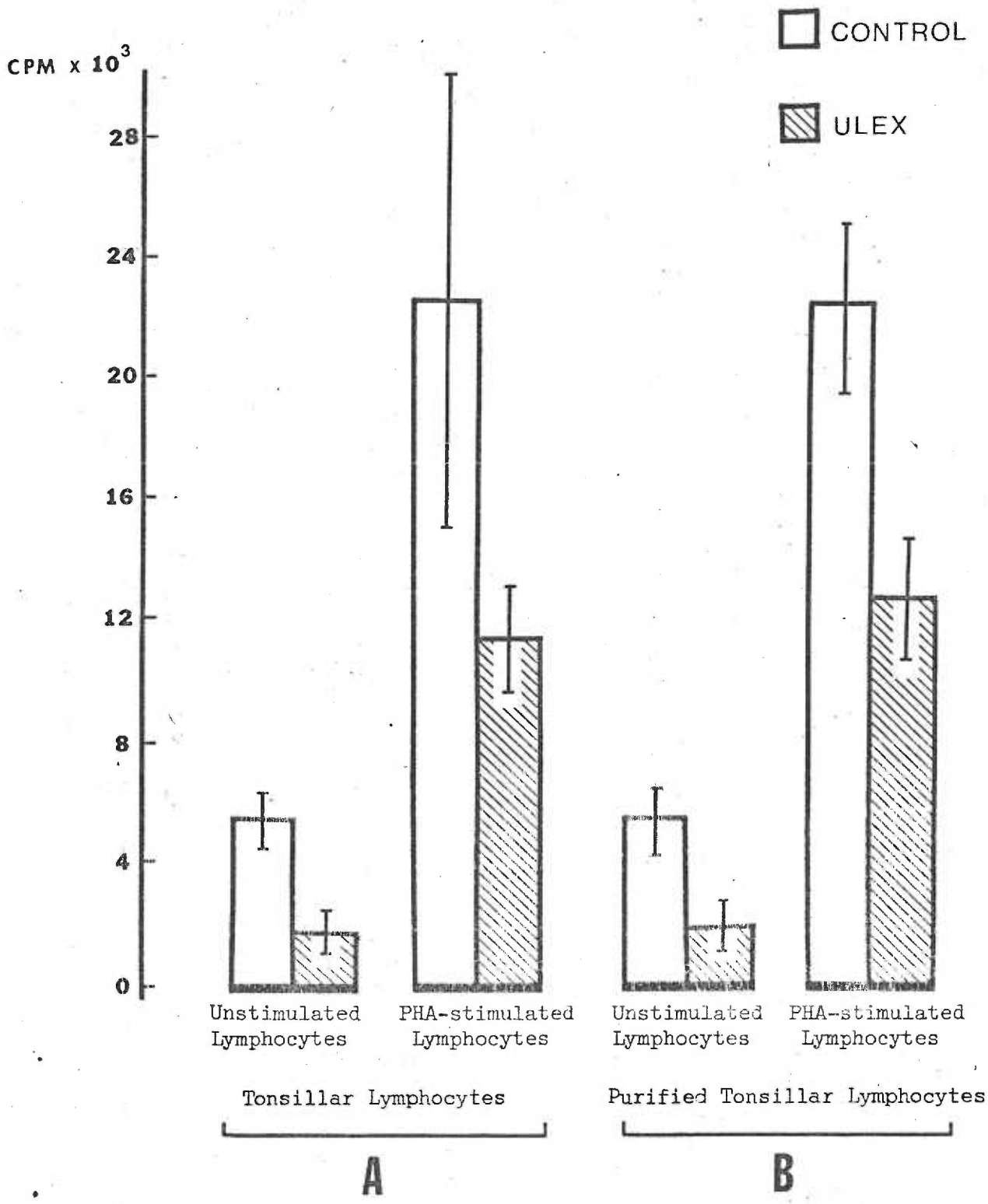
PHA-transformation experiments were performed with human thymocytes, a relatively pure source of T-cells. The results are presented in Figure XVI.

While unstimulated thymocytes are not affected by Ulex europeus

Figure XVII

Effect of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of unstimulated and PHA-stimulated lymphocytes. (A) Tonsillar lymphocytes containing both B and T-cells. (B) Purified tonsillar lymphocytes containing only lymphocytes with no complement-receptor sites or T-cells. (Mean CPM ± 1 S.D.)

Figure XVII


CONTROL
ULEX
CPM x 10³
28
24
20
16
12
8
4
0
Unstimulated Lymphocytes
PHA-stimulated Lymphocytes
Unstimulated Lymphocytes
PHA-stimulated Lymphocytes
Tonsillar Lymphocytes
Purified Tonsillar Lymphocytes
A
B

seed extract, PHA-stimulated thymocytes show a significant reduction ($p < .01$) of DNA synthesis in the presence of the plant extract. The low level of tritiated thymidine incorporation is not disturbing. Thymocytes are known to respond to PHA to a lesser extent than peripheral blood lymphocytes. The discrepancy is usually explained by the presence within the thymus gland of at least two subpopulations of lymphocytes.

In order to reduce the difficulty of dealing with more than one functional population of lymphocytes, tonsillar lymphocytes were purified to remove B-cells. Cells so obtained were considered as representative of the peripheral T-cell population. Unpurified tonsillar lymphocytes and tonsillar lymphocytes not containing complement receptor sites were cultured with and without PHA in the presence of Ulex europeus seed extract. Results are presented in Figure XVII. Unpurified tonsillar lymphocytes and tonsillar non-complement-receptor lymphocytes are highly affected when exposed to Ulex europeus seed extract. The tritiated thymidine uptake is markedly reduced whether they are stimulated by PHA or not ($p < .01$). The inhibition seen in unstimulated cells is most probably due to the high spontaneous tritiated thymidine incorporation. This, in turn, may be a reflection of two factors: 1) the varying ages of tonsillar lymphocytes as seen in any secondary lymphoid organ and 2) the immune activity in infected tonsils. When stimulated with PHA, these cells undergo transformation to a lesser degree than peripheral blood lymphocytes. This phenomenon is generally explained by the fact that committed cells are less

Figure XVIII

Effect of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of leukemic lymphocytes (CLL) stimulated with pokeweed mitogen. (Mean CPM ± 1 S.D.)

Figure XVIII

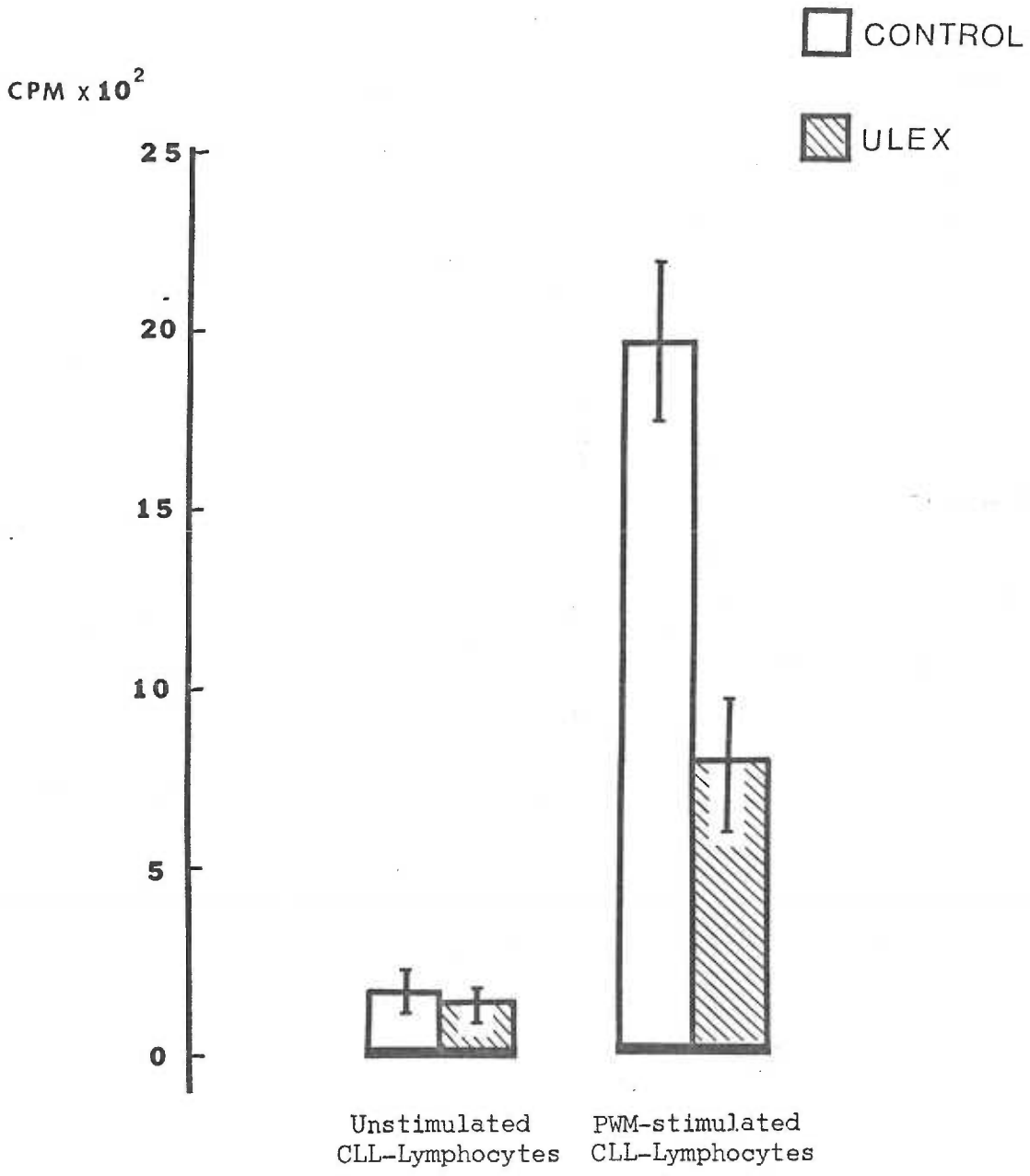
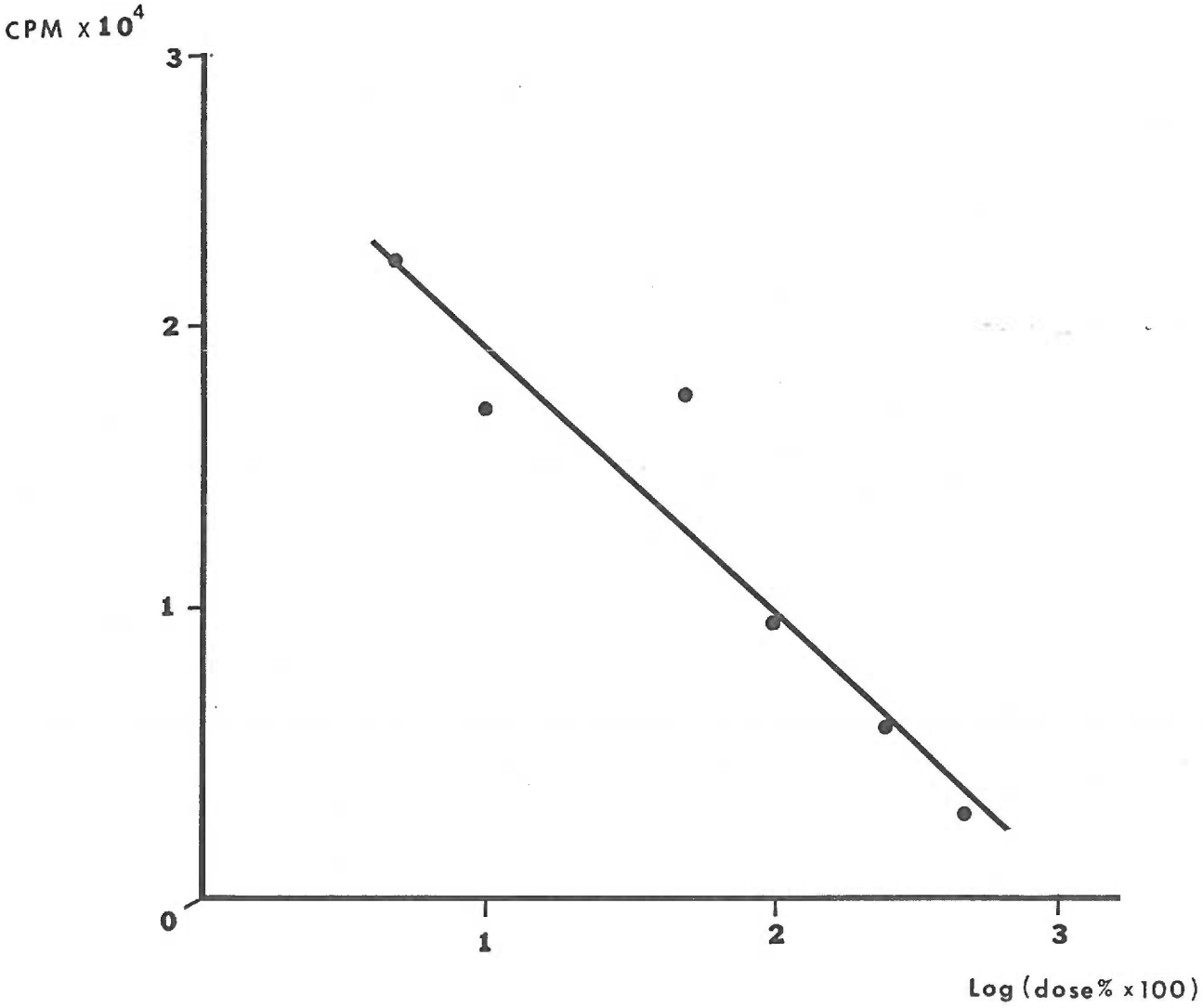


Figure XIX

Effect of several concentrations (V/V) of Ulex europeus seed extract upon the tritiated thymidine uptake of PHA-stimulated lymphocytes. CPM are plotted as a function of the $\log_{10}$ value of the plant extract concentration X 100. The regression line is the best fit for the entire data.

Figure XIX



responsive to the action of other mitogens. Nevertheless, Ulex europeus seed extract still exerts its inhibitory effect on both populations ($p < .05$). Lymphocytes without complement receptor sites are affected to the same extent as unpurified cells obtained from tonsils.

Peripheral blood lymphocytes, obtained from two patients with chronic lymphocytic leukemia contained surface immunoglobulins and were considered as representative of the B-cell population. These cells were studied in PWM-transformation experiments. The results are presented in Figure XVIII. Ulex europeus seed extract did not inhibit tritiated thymidine uptake of unstimulated cells. After PWM-stimulation, these cells were affected in a similar fashion as other lymphocytes ($p < .01$).

Dose effect of Ulex europeus seed extract upon lymphocyte DNA synthesis

Experiments were designed to study the dose-effect of Ulex europeus seed extract. Peripheral blood lymphocytes were stimulated with PHA and exposed to several concentrations (V/V) of the seed extract. Results are presented in Figure XIX. It was demonstrated that inhibition of lymphocyte tritiated thymidine uptake increases according to an arithmetic progression only when the concentration of Ulex europeus seed extract increases according to a geometric progression. This dose-effect response is in accordance with the Fechner-Weber law which characterizes pharmacological effects of drugs. The regression analysis of CPM/log (dose % X 100) is detailed in Appendix II. The regression line presented in Figure XIX is the best fit for the entire data

Table V

Serial agglutination titer of anti-H activity of Ulex europeus seed extract against human erythrocytes of various blood types.

Table V

Cell	DILUTION									Total Score
	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
"O"	12	12	12	12	12	10	5	3	0	78
"A "	12	12	12	12	10	8	0	0	0	66
"B"	10	8	5	3	2	0	0	0	0	28
"A "	5	3	0	0	0	0	0	0	0	8
"O_h " "Bombay"	0	0	0	0	0	0	0	0	0	0

and was plotted according to the following equation: estimated CPM = 2887 - 931 log (dose Ulex europeus % (V/V) X 100).

Effect of anti-H agglutinins upon lymphocyte DNA synthesis

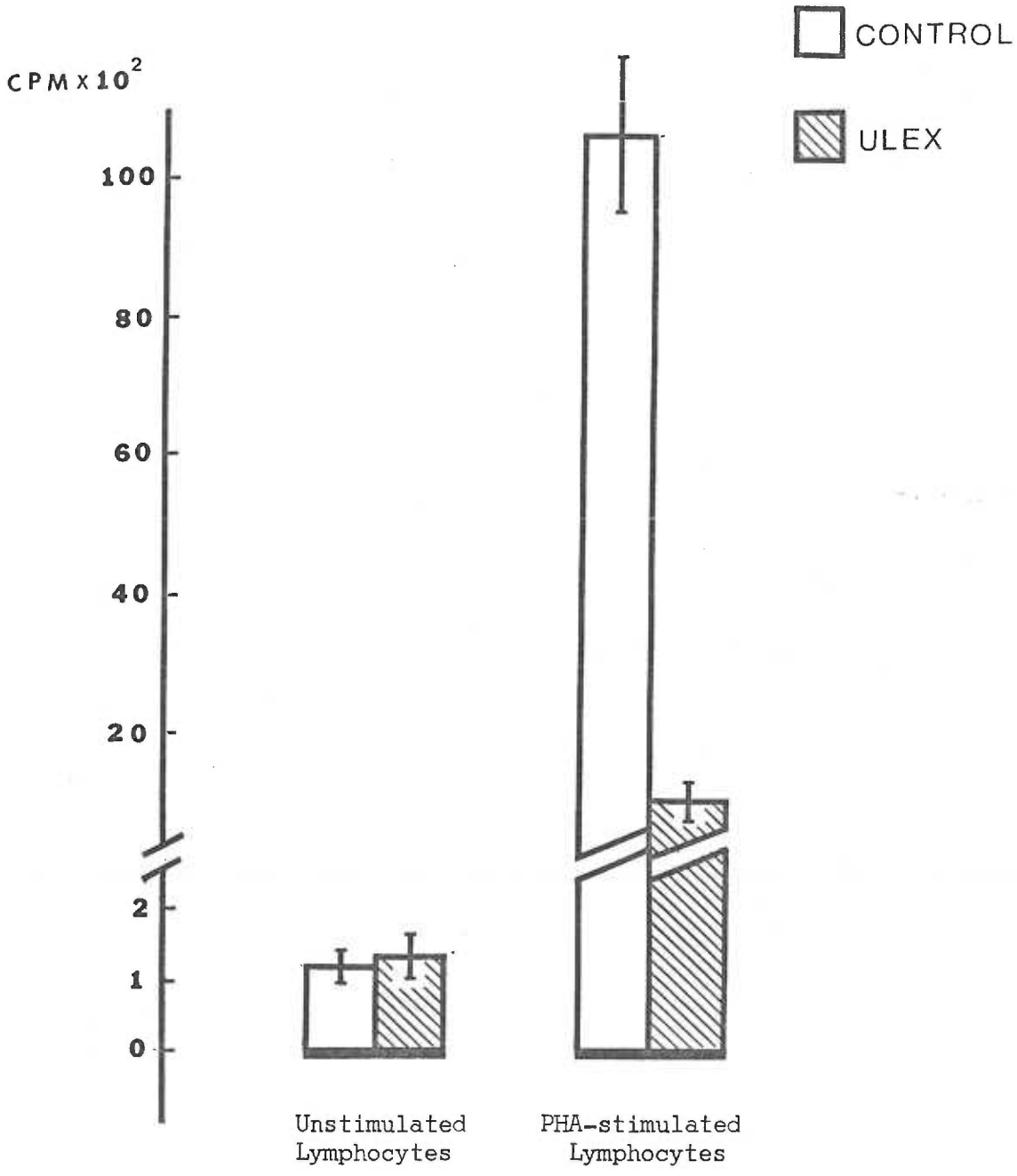
In order to investigate if anti-H agglutinin present in Ulex europeus seed extract was the active material, titration of anti-H activity was performed against a panel of fresh human A, B and O erythrocytes. Table V summarizes the results of red cell agglutination. Scores indicate a high correlation between the amount of H substance present on the red cell membrane and the activity of Ulex europeus seed extract. Erythrocytes of blood group "O" react strongly with the seed extract while those of blood group A_1 show minimal agglutination. Since red cells of blood group "Bombay" do not have any H substance, their lack of agglutination in this system was expected. Lymphocyte agglutination was not observed when similarly tested.

The relationship of the anti-H activity of Ulex europeus seed extract to its lymphocyte inhibition was investigated. The seed extract was neutralized with saliva containing H substance, obtained from blood group "O" secretors. Peripheral blood lymphocytes were stimulated with PHA and the neutralized extract evaluated for their capacity to inhibit DNA synthesis. Since saliva from non-secretors was not available, results were interpreted with the use of the multifactorial analysis in order to discriminate effects of each factor involved and their interactions upon observed tritiated thymidine uptake. It was found that saliva and Ulex europeus seed extract, individually had an

Figure XX

Effect of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of unstimulated and PHA-stimulated peripheral blood lymphocytes from an individual of blood group "Bombay." (Mean CPM ± 1 S.D.)

Figure XX


CONTROL
ULEX
CPM x 10²
100
80
60
40
20
2
1
0
Unstimulated Lymphocytes
PHA-stimulated Lymphocytes

inhibitory effect upon the lymphocyte thymidine incorporation ($p < .01$). When combined, i.e., after neutralization of anti-H, the results could not be explained by the summation of the effects. Ulex europeus seed extract, after neutralization with saliva, was less inhibitory to lymphocyte thymidine uptake than unneutralized extract ($p < .01$). These results suggest that saliva of blood group "O" secretors contain a substance or substances which reduces the inhibitory activity of Ulex europeus seed extract upon lymphocyte metabolism. Details of computational procedures are presented in Appendix III.

This observation does not prove that the anti-H agglutinins are the inhibitory material present in Ulex europeus seed extract. Saliva is known to contain numerous antigenic materials other than H substance. In an attempt to clarify this point, lymphocytes obtained from an individual of blood group "Bombay" were assayed with Ulex europeus seed extract in a PHA transformation experiment. Results are presented in Figure XX. Unstimulated lymphocytes are not affected by the seed extract. However, PHA-stimulated lymphocytes are inhibited to the same extent as lymphocytes of the ABO blood groups ($p < .01$). These findings suggest that the inhibitory material present in crude extract of Ulex europeus seed is not related to anti-H. This is based on the assumption that the inherited disorder of blood group "Bombay" i.e., an absence of erythrocyte H substance, extends to other cells such as lymphocytes.

Effect of ulexine (cytisine) upon lymphocyte DNA synthesis

Figure XXI

Effect of ulexine (cytisine) at several concentrations upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

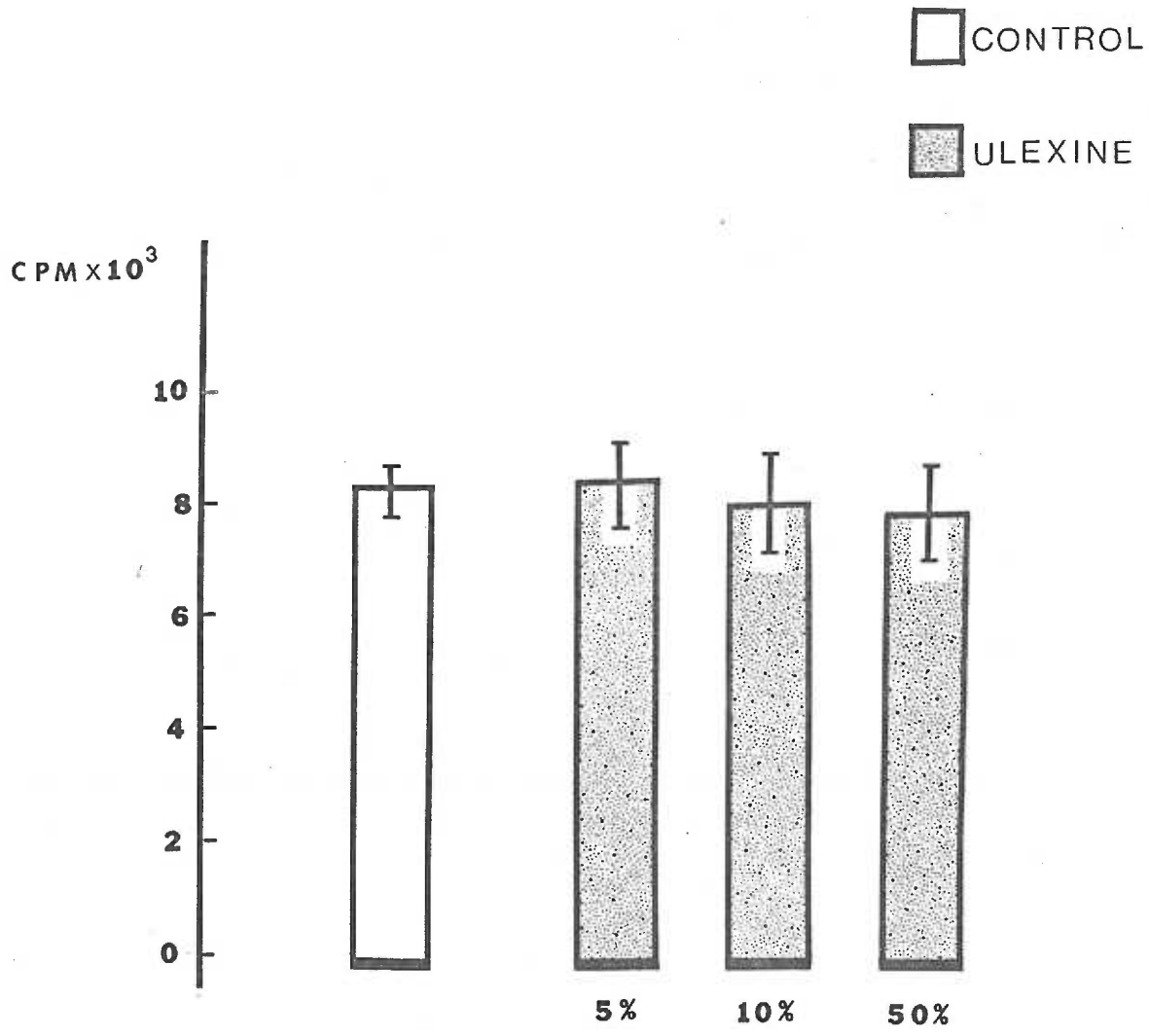
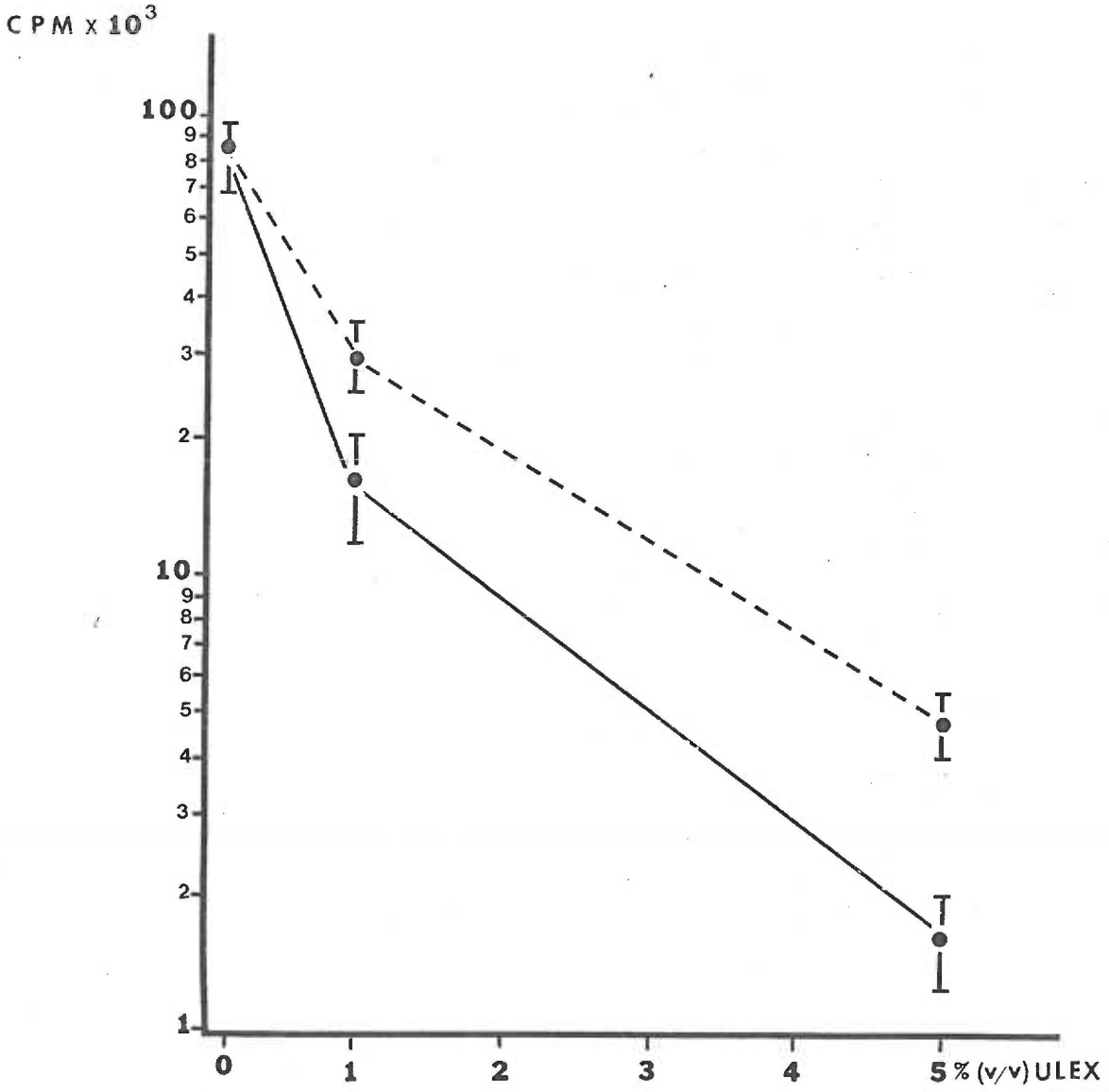

CONTROL
ULEXINE
CPM x 10³
10
8
6
4
2
0
5%
10%
50%

Figure XXII

Effect of Ulex europeus seed extract at a concentration of 1 and 5% (V/V) upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. ●——● represents crude extract and ●----● represents heated extract. (Mean CPM ± 1 S.D.)

Figure XXII



As previously indicated, Ulex europeus seed extract is known to contain an alkaloid material termed ulexine (but generally called cytisine). Studies were performed to evaluate the potential role of this material on the lymphocyte inhibitory action of the seed extract. At concentrations corresponding to the ulexine content of 5%, 10% and 50% (V/V) of the crude extract as evaluated by gas chromatography and ultraviolet absorption spectrometry, the alkaloid material was incubated with peripheral blood lymphocytes undergoing PHA transformation. Results are presented in Figure XXI. There was no significant difference between lymphocytes exposed to ulexine and controls. It may therefore be assumed that the alkaloid content is not responsible for the thymidine uptake inhibition capacity of Ulex europeus seed extract.

Physico-chemical characteristics of the inhibitory substance(s)

Preliminary studies were made to characterize some of the physico-chemical properties of the inhibitory present in Ulex europeus seed extract. Several fractionation procedures were utilized as described in Materials and Methods. The fractions of Ulex europeus seed were assayed in PHA-transformation experiments using peripheral blood lymphocytes.

In order to study the effect of heat on the inhibitory material, experiments were performed with the heated extract at a final concentration of 1 and 5% (V/V). Results are presented in Figure XXII. It was observed that Ulex europeus seed extract strongly inhibits lymphocyte thymidine uptake after heating ($p < .01$), but that the inhibitory activity is less than the native extract ($p < .05$). These results

Figure XXIII

Effect of Ulex europeus seed extract at a concentration of 1 and 5% (V/V) upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. ●——● represents crude extract, and ●----● represents crude extract dialyzed against phosphate buffer. (Mean CPM ± 1 S.D.)

Figure XXIII

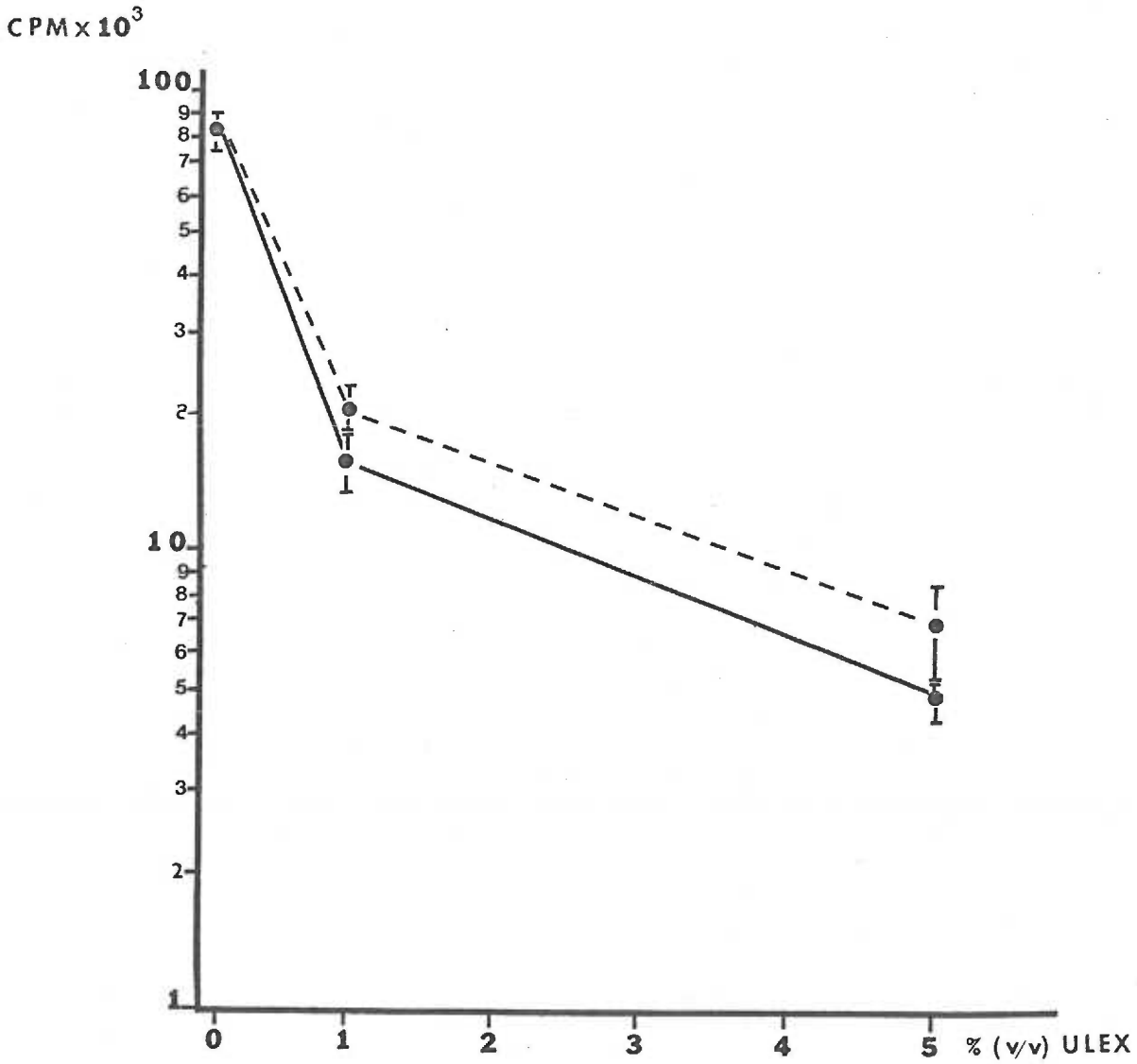


Figure XXIV

Effect of Ulex europeus seed extract and its TCA-soluble and insoluble fractions at a concentration of 1 and 5% (V/V) upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

Figure XXIV

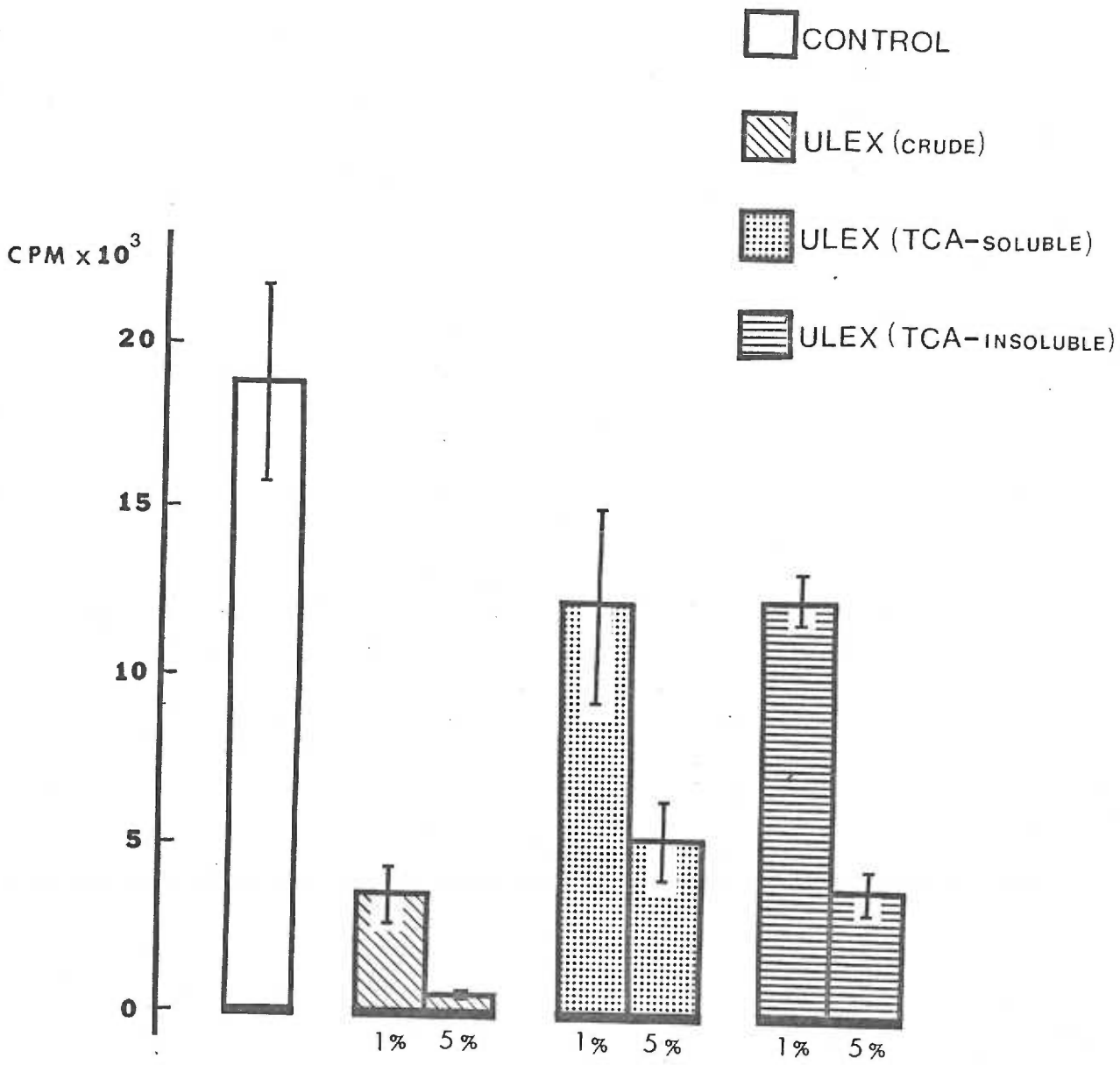
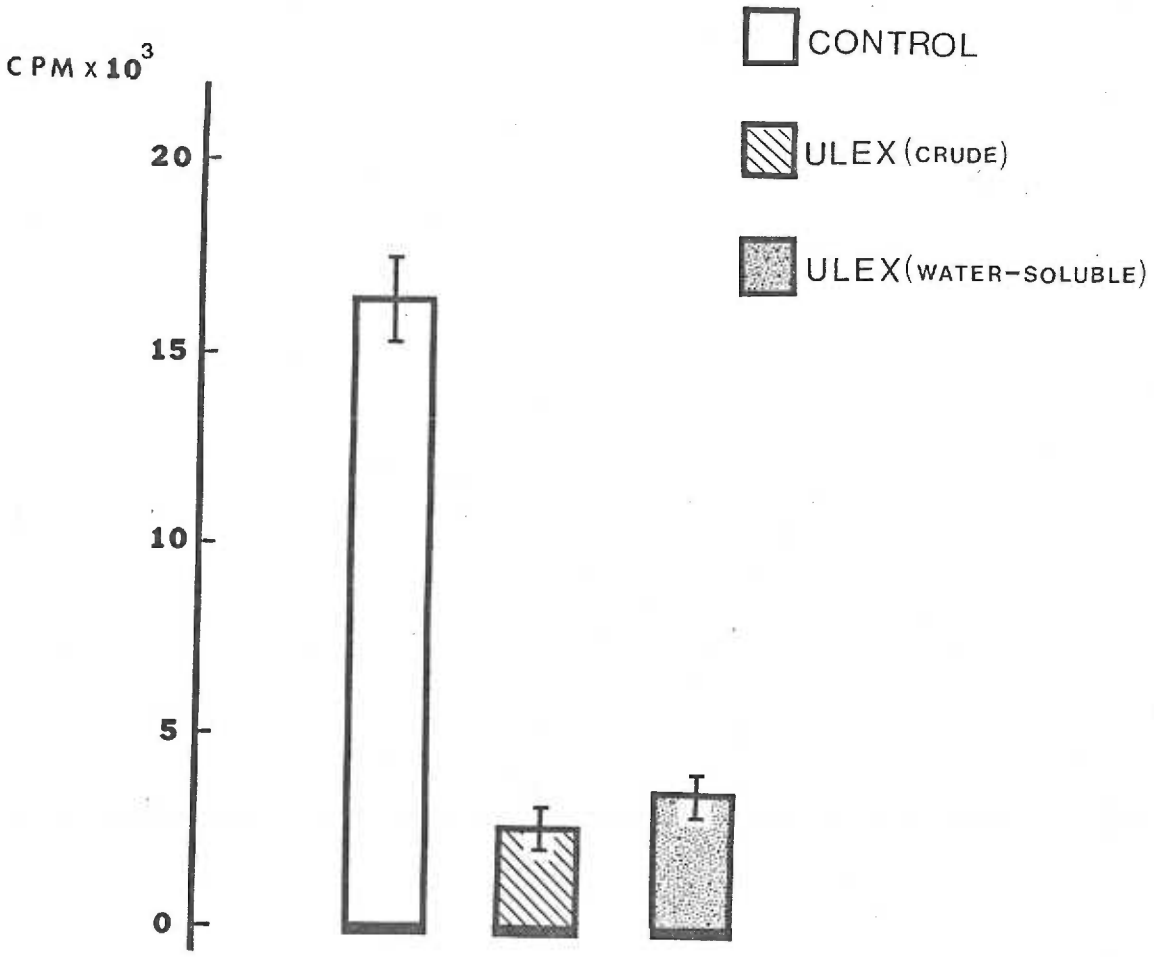

CONTROL
ULEX (CRUDE)
ULEX (TCA-SOLUBLE)
ULEX (TCA-INSOLUBLE)
CPM x 10^3
20
15
10
5
0
1%
5%
1%
5%
1%
5%

Figure XXV

Effect of Ulex europeus seed extract [1% (V/V)] before and after dialysis against distilled water, upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. (Mean CPM ± 1 S.D.)

Figure XXV



suggest that either the active material is relatively heat-stabile or has been partially removed nonspecifically with the precipitate. Crude extract of Ulex europeus seed was dialyzed against phosphate buffer and the dialyzed extract was assayed in order to roughly determine molecular size of the inhibitory material. Figure XXIII summarizes the results. It was observed that the lymphocyte metabolism inhibitor is not dialyzable. Small molecular weight substances such as glycine which was used to stabilize the extract, are not involved in the metabolism inhibition.

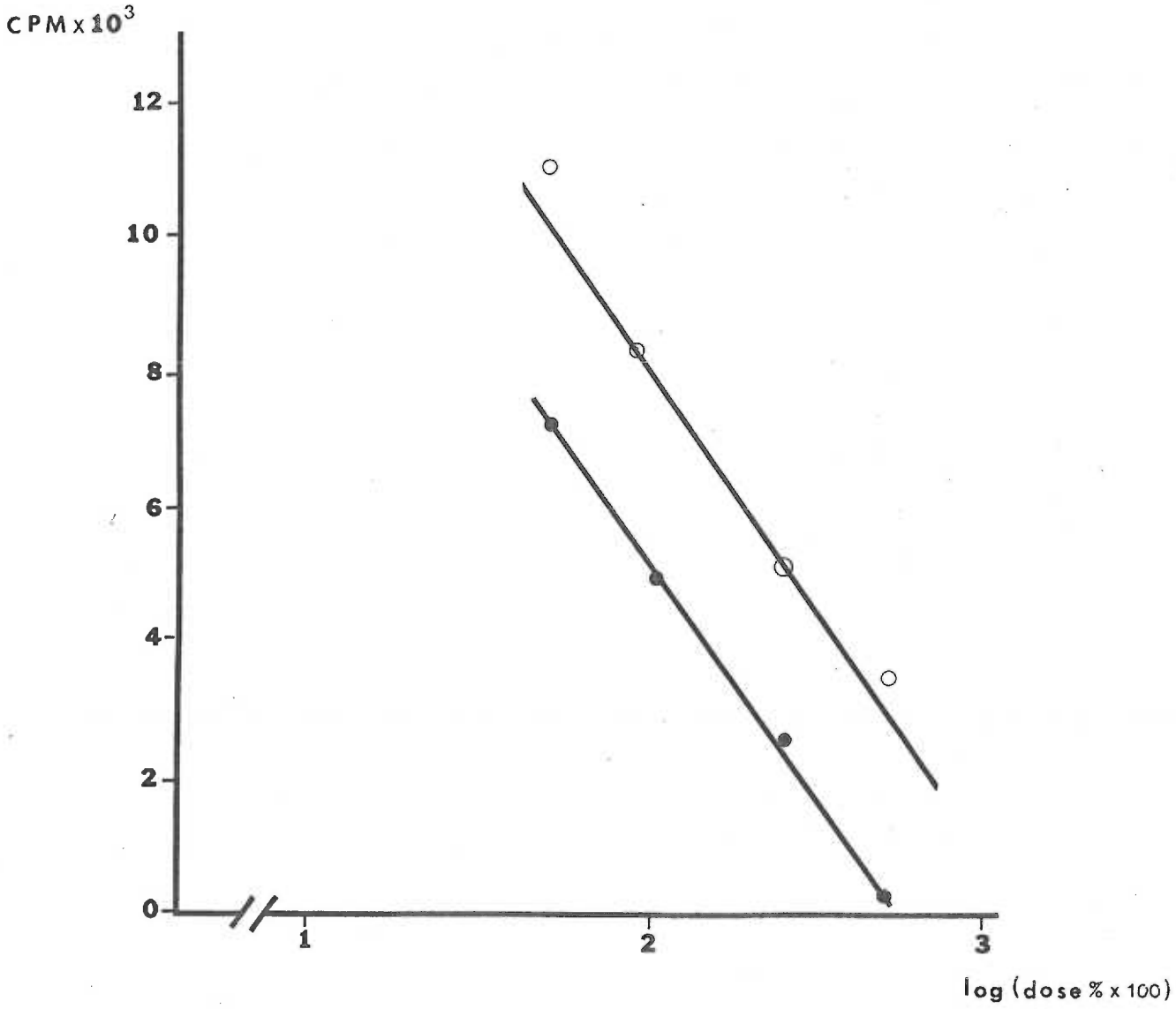
Attempts were made to separate the active material with the use of trichloroacetic acid (TCA). There was no significant difference between the activity of the precipitate and the residual soluble fraction (Figure XXIV). The possibility of a nonspecific co-precipitation has been considered. Multiple washings of the insoluble fraction could not remove any inhibitory activity. Since both fractions remain active ($p < .01$), it indicates that the inhibitory material is more complex than a simple protein.

In order to determine if the inhibitory material was soluble at very low ionic strength, crude extract of Ulex europeus seed was dialyzed against distilled water. A precipitate occurred. It was observed that the active material was only present in the supernatant. Figure XXV records the lymphocyte inhibitory effect of water soluble fraction of Ulex europeus seed extract.

A small but constant difference was seen in the lymphocyte thymidine uptake of cultures exposed to the crude extract and the extract

Figure XXVI

Effect of several concentrations (V/V) of Ulex europeus seed extract before (●) and after dialysis against phosphate buffer (O), upon the tritiated thymidine uptake of PHA-stimulated peripheral blood lymphocytes. CPM are plotted as a function of the $\log_{10}$ values of the final concentration (V/V) X 100 of the seed extracts. Regression lines are the best fits for both groups of data.


CPM x 10³
12
10
8
6
4
2
0
1
2
3
log (dose % x 100)

dialyzed against phosphate buffer. Further studies were performed to determine if this represents a real loss of activity or is due to variation within the test system. Peripheral blood lymphocytes from one individual were stimulated with PHA and exposed to several concentrations of the crude extract and the dialyzed extract of Ulex europeus seed. Results are presented in Figure XXVI. Both extracts inhibit strongly the lymphocyte thymidine uptake and obey the Fechner-Weber law. However, the crude extract is more potent than the dialyzed extract in inhibition capacity. Statistical analysis of both curves demonstrates that the crude extract is at least 1.74 and not more than 2.76 more inhibitory than the dialyzed extract at a level of probability of 5%. Appendix IV illustrates the computational procedures of the analysis of regression for both curves and the inhibitory activity of the respective extract.

DISCUSSION

The results presented in this thesis indicate that an extract of Ulex europeus seeds inhibit lymphocyte DNA and protein metabolism. This conclusion is based on the marked reduction of thymidine and leucine uptake of PHA-stimulated peripheral blood lymphocytes, when exposed to Ulex europeus seed extract. The observations that pokeweed-stimulated lymphocytes and lymphocytes involved in mixed lymphocyte reactions are similarly affected, indicate that the inhibition observed is not secondary to competition between PHA and Ulex europeus seed extract.

It is known that a specific receptor site exists on lymphocytes for the mitogenic principle of PHA (104-105). In addition, initiation of immune responses involve the recognition of antigen by specific receptors on the surface of lymphocytes. These receptors are immunoglobulin-like molecules. Pre-treatment of lymphocytes with anti-heavy and anti-light chain sera blocks the subsequent binding of antigen to the cell surface (106). Antibody to light chain suppresses the ability of mouse spleen or peritoneal exudate cells to induce the graft-versus-host reaction or the transfer of delayed hypersensitivity (107). PHA receptor sites and antigen recognition receptor sites are accordingly different and it appears unlikely that Ulex europeus seed extract can nonspecifically block all these specific sites. This hypothesis is supported by the fact that untreated lymphocytes not specifically stimulated are also affected by the plant extract.

It appears to be most probable that Ulex europeus seed extract acts directly upon lymphocyte metabolism through its own pathway, rather than interfering with the various stimulatory substances.

The inhibitory capacity of Ulex europeus seed extract is not due to irreversible cytotoxicity directed against lymphocytes. Exposure of whole blood to a 33% (V/V) concentration of Ulex europeus seed extract does not induce deleterious effects upon lymphocyte reactivity (90). It is true, however, that exposure is generally limited to short periods of time and that lymphocytes are freed from the extract by washing before study. Experiments performed as part of this thesis have clearly demonstrated that even prolonged exposure to Ulex europeus seed extract at a concentration of 5% (V/V) does not affect lymphocyte membrane permeability as evaluated by dye exclusion and chromium release techniques. The return of lymphocytes to normal reactivity after the removal of Ulex europeus from the tissue culture medium indicates the reversibility of the inhibition. This finding suggests that the interaction of the plant extract with the lymphocytes is not one of tight affinity and is unlikely to involve an intracellular receptor.

A mitogen is usually evaluated by its ability to induce lymphocyte blastogenesis, and its potency measured by the amount of newly synthesized material. Ulex europeus seed extract was studied in this regard by its capacity to block this action. It was observed that the higher the lymphocyte metabolism and the faster the mitotic cycle,

the greater was the inhibition produced by the plant extract. While unstimulated peripheral blood lymphocytes were mildly affected, PHA stimulated lymphocytes or lymphoblasts derived from Itwazuki cell-line were markedly inhibited.

The number of large cells and mitoses arising spontaneously in cultures of tonsillar lymphocytes has been reported as greater than those of control cultures of peripheral blood lymphocytes (108). Since tonsils represent lymphoid tissue exposed at all times to external antigens, the apparent spontaneous change may be the result of stimulation initiated in vivo (109). It must also be considered that tonsils represent a secondary (if not a primary (110)] lymphoid organ with numerous young active cells (111). This active metabolism as observed in unstimulated cells was also depressed by Ulex europeus seed extract as shown in Figure XVII. Accordingly, these observations suggest that the inhibition exerted by Ulex europeus seed extract on lymphocyte metabolism is directly related to the metabolic activity of the cells, regardless of the stimuli initiating their responses. In addition, it was found that the degree of lymphocyte metabolic inhibition was also related to the length of exposure to the plant extract as illustrated in Figure VII. This observation rules out the possibility that crude extract of Ulex europeus seed contains small nucleotides or thymidine-catabolic enzymes such as thymidine phosphorylase which might lead to spurious low values of tritiated thymidine incorporation. A gross contamination would show equally low values, independently of the length of exposure to the

extract and a weak contamination would affect only cultures pulsed prior to harvesting. Indeed, any trace of nucleotides or thymidine catabolic enzymes present in the extract would be utilized in the course of PHA-transformation and would have no effect on tritiated thymidine pulse before the harvesting. Counts Per Minute would be inversely related to the length of exposure to the extract.

All these PHA-transformation experiments were performed by exposing lymphocytes to a continuous pulse of PHA. It has been shown that a continuous stimulation is necessary in order to permit the progeny of PHA-stimulated lymphocytes to replicate (112). Despite the fact that all the cells in lymphocyte cultures are exposed to PHA at the same time, the degree of synchronization of subsequent activity is low (113). Even when achieved, it is only temporary since the cell cycle characteristics of a lymphocyte are not usually transmitted to its progeny. After a few generations, the cells will revert to a population with randomized cycle times, similar in mean parameters to the population from which they were originally derived (57). With these facts in mind, it may be hypothesized that Ulex europeus seed extract acts mostly by preventing cell activation. This assumption would explain the low tritiated thymidine uptake observed when PHA and Ulex europeus seed extract are simultaneously added to cultures. If the addition of Ulex europeus is delayed, a number of lymphocytes will be irreversibly committed to differentiation and DNA synthesis. Similar observations have been reported in Con-A-transformation experiments. Addition of methyl-α-D-mannoside (Con-A inhibitor) after 20

hours of incubation with Con-A, did not prevent DNA synthesis (114). The longer the interval between incubation with PHA and the addition of Ulex europeus seed extract, the greater will be the number of cells committed to a mitotic cycle.

What happens to irreversibly stimulated cells after Ulex europeus seed extract has been added? Although the present experiments do not exclude the possibility that some cells might be trapped in the S-phase, it is most likely that such lymphocytes complete one cell division and the daughter cells return to the quiescent state of the original population. This view is supported by the results presented in Figure VI. In this particular experiment, the cells involved were lymphoblasts at the start. Since no activation or differentiation processes are involved, the reduced thymidine uptake of lymphocytes exposed to Ulex europeus seed extract may be explained by a prolonged G_1-phase and a slowing of the mitotic cycle rate. Alternatively, the "mitosis operon" may be turned off and lymphoblasts take the pathway leading to small lymphocyte or G_o-phase. Preliminary data on the morphological appearance of lymphoblasts exposed to Ulex europeus seed extract appear to support this latter view.

Attempts to demonstrate a selective inhibitory activity of Ulex europeus seed extract among various lymphocyte populations were unsuccessful. Thymocytes, non-complement receptor site type lymphocytes, PHA-stimulated peripheral blood lymphocytes and lymphocytes involved in mixed lymphocyte reactions failed to show any major differences in the capacity for Ulex europeus seed extract inhibition. These cells,

representing several varieties of thymus-dependent lymphocytes (115, 48, 45, 116), were consistently inhibited by Ulex europeus seed extract. As sole representatives of the B-lymphocyte population, lymphocytes obtained from patients with chronic lymphocytic leukemia and lymphoblasts derived from the Itwazuki-lymphoid cell line, were also inhibited by Ulex europeus seed extract. Both types of cells have been defined as B-cells by the presence of surface immunoglobulins (89).

A preliminary study was initiated to investigate the nature of the "Ulex seed inhibitor" (USI). Ulexine, a nicotine-like alkaloid, was a primary suspect in view of its well-documented presence in this extract. Several lines of evidence obtained in experimental animals suggest that immunological events might be influenced by the sympathetic nervous system (117). In addition, the demonstration of adrenergic receptors in the human lymphocyte and their apparent role in its metabolism through cyclic adenosine monophophate (cyclic-AMP) and cyclic guanosine monophosphate (cyclic-GMP) have reinforced that concept (118). Accordingly, ulexine could possibly affect lymphocyte metabolism through this pathway. The studies illustrated in Figure XXI, however, appear to rule out an active participation of ulexine (or cytisine) in the inhibitory action. These negative results are significant and valid. The material studied contained cytisine as shown by the Munier-Macheboeuf-Dragendorf's reagent and later confirmed by gas chromatography (the last analysis was kindly performed by Sterling Sorensen, Department of Clinical Pathology, Toxicology Section, U.O.M.S.).

It is also well recognized that Ulex europeus seed extract contains anti-H agglutinins. L-fucose and salicin, inhibitors of anti-H agglutinins, were unfortunately unavailable for neutralization studies. Saliva from H-substance secretors were utilized to neutralize the anti-H_u and anti-H_c activity of Ulex europeus extract (30, 119). The results of this experiment are presented in Appendix III. Initial studies demonstrated that saliva induced the inhibitory activity of Ulex europeus seed extract. This, therefore, suggested that the anti-H activity of Ulex europeus played a role in the inhibition of lymphocyte metabolism. However, saliva contains many materials, including polysaccharide-amino acid-complexes other than H-substance (120). It was obligatory to verify that the H-substance was specifically interacting with the inhibitory material present in Ulex europeus seed extract. An experiment performed with lymphocytes of blood group "Bombay" strongly suggested that this was not the case and anti-H agglutinins were not involved in the saliva-induced negation of inhibition. The results presented in Figure XX indicate that peripheral blood lymphocytes from such rare individuals are affected similar to lymphocytes obtained from subjects of other ABO blood groups. "Bombay" blood group individuals do not have the H gene which specifies the fucosyl transferase; accordingly, they are unable to form the H enzyme in any tissue (121). H-substance is presumably absent from their lymphocytes and the inhibitory activity of Ulex europeus seed extract cannot be explained by its anti-H agglutinin content.

Fractionation procedures also suggest that anti-H agglutinins are probably not the "inhibitory factor." While anti-H activity is destroyed by heating Ulex europeus seed extract at 100°C for 30 minutes, its inhibitory activity upon lymphocyte metabolism remains essentially unchanged. In addition, after treatment with trichloroacetic acid the "inhibitory factor" is recovered in both fractions, i.e., in the TCA-soluble and the TCA-insoluble fractions, while anti-H activity is partially recovered only in the insoluble fraction. It has been shown that Ulex europeus seed extract behaved as large molecular cold agglutinins and also like low molecular weight serum antibodies. On the basis of equilibration studies, Ulex europeus seed extract possesses a heterogeneous molecular population. Some produce readily dissociable agglutinates while others seem to be irreversibly combined with the "antigens" (122). The observations made after trichloroacetic acid fractionation may be explained on this basis. While high molecular weight anti-H agglutinins are precipitable due to a larger protein backbone, anti-H agglutinins of smaller size could remain in solution when TCA is added. This is probably due to a higher carbohydrate-protein ratio. This last fraction, unable to agglutinate red blood cells, would nevertheless be capable of binding to the "antigenic sites." In immunological tests it would resemble a "blocking antibody."

By analogy, it may be hypothesized that Ulex seed inhibitor (USI) exists as a population of glycoproteins of several molecular sizes, differing mostly by the protein content of their backbone structure.

The dialysis studies favor this hypothesis. The results presented in Figure XXVI indicate that some activity of the crude extract is lost during dialysis suggesting an inhibitor or inhibitors of varying molecular weights. Loss of activity secondary to dilution, denaturation due to slight changes of ionic strength or even removal of unidentified potentiates were considered and appeared unlikely. In addition, the concept of micro-heterogeneity of the polysaccharides for several glycoproteins, as developed by Cunningham, may explain why similar effects are observed with different molecules (123).

How does USI act upon lymphocyte metabolism? The present work does not answer this critical question. However, several hypotheses can be suggested on the basis of these results and from the work of others (124, 125). Ulex europeus seed extract antagonism of lymphocyte metabolism may result from interaction of the USI with some receptor located on the lymphocyte membrane. Previous experiments performed with stimulated and unstimulated lymphocytes indicate that there is a noncompetitive interaction between the stimuli and the USI. This interpretation implies the existence of at least two receptors, one for the USI which turns off cell metabolism and one or more receptors for mitogens. Activation of the USI-receptor prevents the metabolic events which mitogen-activated receptor would normally produce. However, the USI produces its effect only as long as the weakly bound USI remains fixed to the cells. There is good evidence that the biochemical events occurring within the membrane at the time of lymphocyte activation, are grossly identical whether the stimulation

is produced by nonspecific or specific mitogen (57). These changes are followed by biochemical and morphological transformation within the cytoplasm, and shortly after by changes in the chemistry of the basic proteins of the chromosomes (126). Since most, if not all, of the newly synthesized material within a cell is influenced by information coming from the nucleus via the DNA-RNA pathway, the effect of the USI may be explained in two ways. On one hand, it is possible that Ulex europeus seed extract impairs the early changes occurring in the membrane following activation, in a similar fashion to chloroquine and prednisolone (127). These two agents stabilize the lysomal membrane and since PHA and other blastogenic agents produce a release of enzymes from lysosomes, it is conceivable that by preventing the breakdown of existing cellular RNA, lymphocytes remain in a quiescent state of metabolism (128). On the other hand, the observation that lymphoblasts are equally turned off in the presence of Ulex europeus seed extract suggests that the nucleus is directly informed of the presence of the USI.

Theoretically, any stimulated lymphocyte and, in particular, B-cells, may act in any one of three ways. They may undergo mitosis, following the clonal theory of Burnet, in a long process dominated by a sequence of specialized synthesis which appear gene-controlled (129). They may "rest," not take part in specialized syntheses and become a memory cell. Finally, they may enter the post-mitotic phase of maturation and proceed toward cellular death actively synthesizing, under gene control, specialized cell products required for their

function. For example, a B-lymphocyte will undergo plasma-cell differentiation while synthesizing immunoglobulins (130). Current data and concepts suggest that there may be two alternative gene-controlled programs (131). A lymphocyte emerging from mitosis may take one of two pathways. These cells may prepare for mitosis by synthesizing the necessary enzymes or undergo changes leading to "rest." This period of choice has originally been called the dicho-phase (124). The USI present in Ulex europeus seed extract, by binding to lymphocyte membrane receptors, may function by switching off the mitotic genes; this effect may persist as long as the USI-receptor complexes are present on the cell membrane.

Obviously, these proposed mechanisms of action are purely speculative and specific data and proof are needed. However, the current studies indicate that although certain plant extracts are lymphocyte-stimulatory, other plant extracts are lymphocyte-inhibitory.

These results suggest that Ulex europeus seed extract contains a substance or substances which are the counterpart of the well-known stimulatory substances present in other plant extracts.

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Appendix I

Effect of Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of PHA-stimulated lymphocytes. Data of Figure II were studied with the use of the analysis of variance applied to single-factor experiment having repeated measures on the same elements.

Appendix I

Person	Control	Ulex europeus	Total
1	37575	6526	44101
2	18206	6258	24464
3	20904	12727	33631
4	22088	12898	34986
5	19876	12094	31970
6	109233	15889	125122
7	15824	3401	19225
8	5089	1229	6318
16	248797	71022	319817

$$(1) = \frac{(319817)^2}{16} = 6,392,682,093$$

$$(2) = (37575)^2+(18206)^2...+(1229)^2 = 16,093,285,155$$

$$(3) = \frac{(248797)^2+(71022)^2}{8} = 8,367,884,563$$

$$(4) = \frac{(44101)^2+(24464)^2+...+(6318)^2}{2} = 10,992,781,693$$

Partition of variation

Total variation	(2)-(1)	= 9,700,603,062
Variation between person	(4)-(1)	= 4,600,099,600
Variation within person	(2)-(4)	= 5,100,503,462
Variation due to Ulex	(3)-(1)	= 1,975,202,470
Residual variation	(2)-(3)-(4)+(1)	= 1,332,718,499

Appendix I - continued

Analysis of variance

Source of Variation	S.S.	d.f.	M.S.	F	p.
Total	9,700,603,062	15	-		
Between Persons	4,600,099,600	7	-		
Within Person	5,100,503,462	8	-		
Ulex	1,975,202,470	1	1,975,202,470	10.37	<.05
Residual	1,332,718,499	7	190,388,357		

Appendix II

Effect of several concentrations of Ulex europeus seed extract upon the tritiated thymidine uptake of PHA-stimulated lymphocytes. Data of Figure XIX were studied with the use of the analysis of regression.

Appendix II

	0.05%	0.1%	0.5%	1%	2.5%	5%
	29700	18320	12890	9020	8070	3270
	17810	24120	14530	8860	5590	3980
	21910	14760	13030	10420	4834	2100
	15620	16120	23040	8580	8180	3180
	26710	13230	24780	9220	4660	1230
$\bar{X}$	22350	17290	17650	9220	6260	2750
ΣX	111750	86550	88270	46100	31310	13760
ΣX^2	26367427 X 10^2	15701417 X 10^2	16919439 X 10^2	4270612 X 10^2	2080075 X 10^2	425686 X 10^2

Total N = 30

ΣX = 377740

ΣX^2 = 65,764,656 X 10^2

Analysis of Variance

Total Variation	S.S.	d.f.	M.S.	F	p
	18,202,153	39	-	-	-
Variation due to Ulex eur.	14,568,340	5	2,913,668	27.27	<.01
Residual Variation	3,633,813	34	106,877	-	-

Analysis of Regression

log (0.05% X 100)	= 0.69897	dose = X
log (.1% X 100)	= 1.00000	CPM = Y
log (0.5% X 100)	= 1.69897	
log (1.0% X 100)	= 2.00000	
log (2.5% X 100)	= 2.39794	
log (5.0% X 100)	= 2.69897	

$$\Sigma x^2 = \frac{(0.69897 \times 5)^2}{5} + \ldots + \frac{(2.69897 \times 5)^2}{5} - \frac{(52.5)^2}{30} = 15.22$$

$$\Sigma xy = \frac{(0.69897 \times 5 \times 111750)}{5} + \ldots + \frac{(2.69897 \times 5 \times 13760)}{5} - \frac{(52.5 \times 377740)}{30} =$$

$$= -14167.5$$

	S.S.	d.f.	M.S.	F	p
Variation due to Ulex eur.	14,568,340	5	2,913,668	27	<.01
Variation due to regression (linear term)	13,187,783	1	13,187,783	123	<.01
Variation due to non-linear term	1,380,557	4	345,139	3.2	N.S

Calculation of the Complete Equation

$$y = a + bx$$

$$b = \frac{-14167.5}{15.22} = -931$$

$$a = 1259 - (-931 \times 1.75) = 2887$$

$$\hat{y} = 2887 - 931 \log (\text{dose \% V/V} \times 100)$$

Appendix III

Effects of saliva from blood group "O" secretor individuals, PHA and Ulex europeus seed extract [1% (V/V)] upon the tritiated thymidine uptake of peripheral blood lymphocytes. Data were studied with the use of the multifactorial analysis.

Appendix III

	No PHA		PHA	
	No Ulex eur.	Ulex eur. 1% (V/V)	No Ulex eur.	Ulex eur. 1% (V/V)
No Saliva	881	89	50735	994
	465	125	52603	1112
	847	100	51486	1121
	581	97	54846	1295
	448	104	45058	982
Saliva	363	410	18808	9054
	699	455	23351	11763
	403	245	17601	16422
	462	185	38178	10431
	658	244	26361	8049

Appendix III (continued)

Multifactorial analysis of variance

Total Variation	S.S.	d.f.	M.S.	F	p
	974,788,687,378	39	-	-	-
Variation due to					
Saliva (A)	159,308,748	1	159,308,748	13.88	<.01
Ulexene (B)	248,497,260,623	1	258,497,260,623	225	<.01
PHA (C)	467,400,618,303	1	467,400,618,303	407.	<.01
Interactions					
A + B	830,877,825	1	830,877,825	72.30	<.01
A + C	162,413,030	1	162,413,030	14.15	<.01
B + C	246,570,076,504	1	246,570,076,504	214	<.01
A + B + C	800,872,857	1	800,872,857	697	<.01
Residual Variation	367,259,488	32	11,476,859	-	-

Appendix IV

Effect of Ulex europeus seed extract at several concentrations before and after dialysis against phosphate buffer, upon the tritiated thymidine uptake of PHA-stimulated lymphocytes. Data of Figure XXVI were studied with the use of the analysis of variance involving a regression.

Crude Extract

Dose	0.05%	1.0%	2.5%	5.0%	Total
Obs.	7874	4704	2504	220	
	4740	5208	2800	202	
	9529	4645	2742	209	
	7397	5100	2300	379	
	7397	4922	2594	310	N = 20
$\bar{X}$	7397	4916	2588	268	3792
ΣX	36937	24579	12940	1340	75796
ΣX^2	284,700,535	121,062,989	33,647,416	381,486	439,792,326

Dialyzed Extract

Dose	0.5%	1.0%	2.5%	5.0%	Total
Obs.	14268	7507	3912	3020	
	8401	8667	6079	3236	
	10329	5613	6813	2465	
	11524	6480	5185	2239	
	11130	7066	4891	2740	
$\overline{X}$	11130	7066	5376	2740	6578
ΣX	55652	35333	26880	13700	186491
ΣX^2	637,520,342	254,896,463	149,481,060	38,189,042	1,400,898,617

Analysis of Variance

$\Sigma\Sigma X = 207,361$

$\Sigma\Sigma X^2 = 1,519,879,233$

$N = 40$

Total variation	S.S.	d.f.	M.S.	F	p
	444,914,625	39	-	-	-
Variation due to Ulex	403,735,152	7	57,676,450	44.8	<.01
Residual Variation	41,179,473	32	1,286,859		

Analysis of regression of the dialyzed extract

log (5% X 100) = 2.69897
log (2.5% X 100) = 2.39794
log (1.0% X 100) = 2.00000
log (0.5% X 100) = 1.69897

X = dose
Y = CPM

Σx^2 = SS (X) = 2.94

Σxy = SS (XY) = -22498

Σy^2 = SS (Y) = 185,690,618

Variation due to Regression	S.S.	d.f.	M.S.	F	p
(linear term)	172,157,910	1	172,157,910	95.2	<.01
Variation due to non-linear term	13,532,708	2	6,766,353	3.74	N.S.
Residual Variation	28,928,828	16	1,808,052		

Calculated equation

$\hat{y}$ = 23413 - 7652 log (dose X 100)

Analysis of regression of the crude extract

log (5.0% X 100) = 2.69897 X = dose
log (2.5% X 100) = 2.39794 Y = CPM
log (1.0% X 100) = 2.00000
log (0.5% X 100) = 1.69897

Σx^2 = SS (X) = 2.94
Σxy = SS (XY) = -20016
Σy^2 = SS (Y) = 140,290,001

	S.S.	d.f.	M.S.	F	p
Variation due to regression (linear term)	136,272,196	1	136,272,196	177	<.01
Variation due to non-linear term	3,697,634	2	1,848,816	2.4	NS
Residual Variation	12,250,645	16	765,665	-	-

Calculated equation

$$\hat{y} = 18770 - 6808 \log (\text{dose} \times 100)$$

Factorial analysis of both regression lines

Source of variation	Factorial coefficients (K)									
	C_1	C_2	C_3	C_4	D_1	D_2	D_3	D_4	$N\Sigma K^2$	$(\Sigma KT)^2$
Difference between Crude & Dialyzed	-1	-1	-1	-1	+1	+1	+1	+1	40	77,754,534
Combined regression line										
-Linear term	-3	-1	+1	+3	-3	-1	+1	+3	200	319,385,011
-Quadrate term	+1	-1	-1	+1	+1	-1	-1	+1	40	1,559,065
-Cubic term	-1	+3	-3	+1	-1	+3	-3	+1	200	1,491,783
Separated regression line										
-non-parallelism (linear term)	-3	-1	+1	+3	+3	+1	-1	-3	200	1,260,713
-Quadrate term	+1	-1	-1	+1	-1	+1	+1	-1	40	1,017,929
-Cubic term	-1	+3	-3	+1	+1	-3	+3	-1	200	1,266,118

Source of Variation	S.S.	d.f.	M.S.	F	p
Difference between Crude & Dialyzed	77,754,534	1	77,754,534	60.4	<.01
Combined regression line					
- linear term	319,385,011	1	319,385,011	248	<.01
- Quadrate term	1,559,065	1	1,559,065	121	N.S.
- Cubic term	1,491,783	1	1,491,783	1.15	N.S.
Separated regression line					
- linear term	1,260,713	1	1,260,713	.97	N.S.
- Quadrate term	1,017,929	1	1,017,929	.79	N.S.
- Cubic term	1,266,118	1	1,266,118	.98	N.S.
Residual error	41,179,473	32	1,286,859	-	

Activity

Activity of both extracts was calculated according to the following formula (ref. 103):

$$\log \frac{\text{dialyzed extract}}{\text{crude extract}} = \overline{X} \text{ crude} - \overline{X} \text{ dialyzed} + qi \sqrt{\frac{D^2}{L^2}}$$

$$= .342$$

The interval of confidence (95%) was calculated according to the following formula (ref. 103):

$$\text{S.E. of } \log \frac{\text{dialyzed extract}}{\text{crude extract}} = \frac{qi \sqrt{(D^2 + L^2)(E^2)}}{L^2}$$

$$= .0494$$

$$\log \frac{\text{dialyzed}}{\text{crude}} = .342 \pm \text{"}\tau\text{"} .0494$$

$$= 1.74 \text{ and } 2.76$$