The in-vitro response of bovine peripheral blood lymphocytes to phytohemagglutinin and tuberculin

H. David Kay

Iowa State University

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The *in vitro* response of bovine peripheral blood lymphocytes to phytohemagglutinin and tuberculin

by

H. David Kay

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

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INTRODUCTION

The in vitro activation or stimulation of lymphocytes in the presence of phytohemagglutinin or tuberculin from a "resting" phase into a state of active DNA synthesis ("blast transformation") is a phenomenon currently receiving great attention. Originally reported by Hungerford et al. (1959) and Nowell (1960), such blast transformation has elevated the small lymphocyte from its early relegation as a "cell of mystery", often categorized by many as an "end cell" with no known function, to its present status as one of the most versatile and fascinating cell types in an animal body. The phenomenon of lymphocyte blastogenesis, as induced in vitro by a variety of mitogenic agents, now provides a satisfactory experimental system for the study of problems central to both immunology and cellular differentiation. Men in many fields of research (immunologists, hematologists, pathologists, cell and molecular biologists, radiobiologists, and many others) have become involved in this exciting area of study, finding answers to many of the questions once posed by the lymphocyte. As a result of their work during the past decade, carried out in many research centers the world over, the field of lymphocyte biology has expanded far beyond all expectation. At the present time, the study of lymphocyte function, production, and relationship to other lymphoid cells constitutes one of the most active, rapidly growing, and productive fields in all of immunology.

A great range of substances, from metallic ions to bacterial products, antibodies, and specific antigens, have been shown to activate lymphocytes in much the same fashion as phytomitogens. Much of the impetus for this type of in vitro work came from the early observation that morphological
and biochemical characteristics of mitogen-induced blastogenesis in vitro were very similar to antigen-induced immune transformation in vivo. Coulson and Chalmers (1964b) suggested that the various mitogenic substances somehow bypass the normal "antigenic recognition" step and induce cells to enter that pattern of response ordinarily dependent on strict immunological activation. Consequently, lymphocyte blastogenesis in vitro has been found to offer not only a means of analyzing the biochemical events involved in cellular derepression (Hirschhorn et al., 1969), but it has also been shown to be of great value as a clinical tool for monitoring the immunological competence of lymphocytes from patients who possess various immunological disorders or who are undergoing immunosuppressive therapy (Oppenheim, 1968).

Although the in vitro lymphocyte response may not be regarded as the ideal model for all phases of the normal immune response, it nevertheless seems to adequately represent at least the proliferative phase of the phenomenon. Thus, this in vitro system is also a convenient one for investigating factors which might regulate cellular proliferative capability and, hopefully, may then shed light on factors involved in controlling the growth of tumor cells.

The degree of lymphocyte transformation induced by specific antigens, non-specific mitogens, or histoincompatible lymphocytes may be quantitated by several methods. One procedure is to assess in stained preparations the morphologic transformation of stimulated lymphocytes into large, basophilic blast-like cells. In addition, blastogenesis can be followed by measuring cellular incorporation of radiolabeled precursor molecules into newly synthesized DNA, RNA, and protein molecules. This is achieved using the methods of liquid scintillation and/or autoradiography. The kinetics
of biosynthesis of DNA, RNA, and protein molecules can then be compared to the morphologic observations of transformation.

This dissertation is concerned with the \textit{in vitro} study of normal bovine lymphocytes. Beef and dairy cattle are very important economically and are subject to a great variety of diseases which could result in severe economic loss. Yet normal bovine lymphocytes, which are no doubt immunologically active in combating many of these diseases, have never been extensively studied in cell culture and their \textit{in vitro} culture characteristics compared with the response of lymphocytes from other species. The research presented here has been performed to help fill this void. Only when the behavior and function of bovine lymphocytes under normal conditions is better understood can a satisfactory basis be established for further studies of lymphocyte involvement in bovine health and disease.
LITERATURE REVIEW

The lymphocyte: definition

The lymphocyte today may well be the most intensively studied cell type in the animal body. This cell is receiving major attention from researchers and clinicians, since it has been observed to function in a variety of vital body processes, ranging from antibody production to the immunologic killing of cancer cells.

Hematologists are quite familiar with the lymphocyte as one of the white cells that circulate in the blood, making up about 25% of the blood leukocytes in man and variable proportions in other mammals. The lymphocyte is not, however, strictly a blood cell; it is also the major cell type in the lymph nodes, spleen, thymus, and intestinal Peyer's patches. Lymphocytes are commonly distributed in other tissues as well, such as the bone marrow and intestinal mucosa, and constitute, in toto, a considerable mass of tissues. For example, the human body contains approximately 1,300 g of lymphocytes outside the blood, lymph nodes and bone marrow, 70 g in the bone marrow, 3 g in the circulating blood, and 100 g in lymphatic tissue (Pulvertaft, 1959).

A dictionary definition of a lymphocyte is: "A variety of white blood corpuscle which arises in the reticular tissue of the lymph glands. The nucleus is single and is surrounded by protoplasm which is generally described as nongranular. Two varieties are described: (a) the small lymphocytes, which are about the size of red corpuscles and constitute from 22 to 28 per cent of the white corpuscles; (b) the large lymphocytes which are two or three times larger than the small lymphocytes and contain a
larger proportion of protoplasm. They form about 1% of the white corpuscles." (Dorland's Illustrated Medical Dictionary, 1965).

Historical aspects of lymphocyte research

Before any precise knowledge of lymphocyte function existed, active participation by these cells in a wide variety of inflammatory and immunologic reactions was suspected due to their abundance in the tuberculin reaction, in the stroma of certain tumors, and in chronic inflammatory lesions such as those of syphilis and tuberculosis.

Ever since Ehrlich, towards the end of the nineteenth century, inaugurated the scientific study of hematology by means of the differential staining of the blood cells, much controversy and misunderstanding has surrounded this small cell. It was aptly described by Yoffey (1967), "If one may adapt the description of the spleen attributed to Galen ("Organon Plenum Mysterii"), the lymphocyte has long been the 'Cellula Plena Mysterii'." The full extent of the "mysterium" is only now beginning to unfold.

Only little more than 35 years ago, the famous Johns Hopkins pathologist, Arnold Rich, wrote concerning the then known characteristics of the lymphocyte (Rich, 1936):

"Regarding the lymphocyte, I am sure that all who are engaged in the study and teaching of pathology will agree that the complete ignorance of the function of this cell is one of the most humiliating and disgraceful gaps in all medical knowledge. Produced daily in enormous numbers by a mass of distributed lymphoid tissue which, if gathered together, would form one of the most imposing organs of the body, normally constituting one fourth of the circulating leukocytes and increasing or decreasing markedly in number in various types of infection and in
various pathologic states, these cells must undoubtedly serve the body in a most essential way, and yet no information is possessed regarding their function apart from speculation, based on evidence that is equivocal, to say the least."

Rich goes on to say:

"...(Lymphocytes) can always be observed after a day or two in areas of inflammation, and when the inflammation is produced by bacteria or bacterial products, they are often present in enormous numbers, but there is not the slightest notion of what they are doing there. They phagocytose neither bacteria nor other particulate matter. Congregated often in the more peripheral parts of the lesion, they have the appearance of phlegmatic spectators passively watching the turbulent activities of the phagocytes. Literally, nothing of importance is known regarding the potentialities of these cells other than that they move, and that they reproduce themselves."

The understanding of the lymphocyte came about only slowly as data accumulated which clearly demonstrated the role of the lymphocyte in immunological reactions. Although experimentally studied as early as 1917 by Pappenheimer (1917), the studies of McMaster and his associates in the mid-1930's (McMaster and Hudack, 1935; McMaster and Kidd, 1937) provided the first experimental evidence that cells of the lymphatic system might be sites of antibody production, in contrast to the view generally held at that time that cells of the reticuloendothelial system (the macrophage, especially) formed these substances (Sabin, 1939).

Following a suggestion made earlier in the 1930's by Wiseman (1931) that the lymphocyte was concerned in some way with the handling of foreign protein that entered the tissues undigested, Rich et al. (1939) investigated the nature of the large blastic cells in "acute splenic tumor" of man. They found these cells to be lymphoid in nature and suggested therefore that the lymphocyte was involved in some way with the body's "reaction to
foreign protein." Rich, in a remarkably perceptive statement, observed that these cells were not necessarily lymphoblasts in the sense of being immature lymphoid cells, but that they probably were lymphocytes which were greatly enlarged because they performed some special function.

In 1942, Wintrobe proposed that the lymphocyte was in some way involved in the body's healing process, since the cells were often observed to be greatly increased in numbers during convalescence from infections. Because of their strategic position in lymph nodes, Wintrobe suggested that lymphocytes might play an important role in toxin fixation, and possibly, also, in antibody production.

Ehrich and Harris (1942) provided experimental support for Wintrobe's proposals. They determined the cell and antibody content of lymph entering and emerging from lymph nodes which drained the site of injection of antigen in rabbits, and found that both the lymphocyte number and antibody titer in the efferent lymph were much greater than in the afferent lymph. Dougherty et al. (1944) minced pooled lymph nodes from mice injected with antigens and prepared a cell suspension rich in lymphocytes, from which they extracted antibodies to the antigen used. Harris et al. (1945) provided further evidence of the association of the lymphocyte with the production of antibodies when they showed that these substances could be found in higher concentration in lymphocytes collected from lymph as it emerged from a lymph node draining the site of injection of antigen than in those cells leaving an unstimulated node.

Harris and Harris (1949) performed histochemical studies on the cells from draining lymph nodes and found good correlation between the increased pyroninophilia of these cells (which indicated an increase in cytoplasmic
RNA associated with increased protein production) and an increased antibody production in the cell mass as a whole. Although demonstration of elevated levels of antibody protein in the node was not proof that the antibody was made by the cells present there, Harris and Harris did feel that there was justification in assuming that the lymphocytes were the antibody producers.

Most of the evidence for implicating the lymphocyte as a mediator of immunologic activities had, by this time, come either from careful observations of the timing of certain cellular and humoral events or from observations on the apparent proximity of cellular and humoral activities in the lymph nodes.

However, a greater understanding of lymphocyte function and physiology was not possible until experiments, other than routine lymph node analyses, were performed. Such investigations were not long in coming. By means of cell transfer studies, Merrill Chase and his co-workers suggested that lymphocytes were the carriers of cutaneous tuberculin hypersensitivity (Chase, 1945, 1951) and provided evidence for the cellular nature of tuberculin allergy. Chase found that delayed-type tuberculin allergy could be transferred passively by cell suspensions from highly sensitized hosts, but not by serum or plasma from these same persons.

A similar series of experiments were carried out by Mitchison (1955) who worked in yet another area of immunologic activity, that of tissue transplantation immunity. Mitchison demonstrated that heightened resistance to certain tumor homografts in mice could be transferred to secondary hosts by lymph node cells but not by serum. Such evidence proved that lymphoid cells were immunologically involved in tumor transplantation immunity. Billingham et al. (1954) demonstrated this same phenomenon with regard to passive
transfer of resistance to skin homografts.

In spite of these advances in understanding, the lymphocyte was still the center of much controversy and heated discussion, since its origin and fate were totally unknown. As recently as 1958, an extensive review and discussion of available literature regarding the lymphocyte (Trowell, 1958) generated no new insights into lymphocyte function, other than the very speculative suggestion that this "Cinderella Cell" might serve as a convenient carrier and distributor of nuclear material for other cells. Two completely contradictory schools of thought dominated the ideas of lymphocyte biologists at this time. One school held that the lymphocyte was an unimportant end cell, incapable of further differentiation, as evidenced by the following statement: "The small lymphocyte is a poor sort of cell, characterized by mostly negative attributes; small in size, with especially little cytoplasm, unable to multiply, dying on the least provocation, surviving in vitro for only a few days, living in vivo for perhaps a few weeks" (Trowell, 1958).

The second school held that the lymphocyte might be a totipotent stem cell, that is, "a specialized form of mesenchymal cell in a resting, relatively inactive state, reduced to the smallest possible size for the purpose of easy mobilization and transport through the bloodstream" (Yoffey and Courtice, 1956).

The first major breakthrough in lymphocyte understanding came in 1959 and 1960, and then only really by accident. Hungerford and Nowell, at the University of Pennsylvania School of Medicine, were working with routine leukocyte cultures from human patients, both normal and leukemic. In the course of their study, considerable mitotic activity was observed in the
cultures of normal leukocytes, as well as in the cultures of leukemic blasts (Hungerford et al., 1959). Nowell tentatively identified the large, blast-like dividing cells in the normal cultures as monocytes or large lymphocytes which had become mitotically active in vitro after a 2-day latent period. Ordinarily, normal leukocytes do not divide in the peripheral blood. Further experimentation revealed that an apparently non-physiologic mitogenic agent was responsible for activating this latent mitotic potential of circulating lymphocytes; this agent was a plant extract, phytohemagglutinin (PHA), originally employed for its erythrocyte-agglutinating ability in obtaining leukocytes from whole blood (Nowell, 1960). Thus, astute observation during a routine experiment led to the removal of a major impediment to the understanding of lymphocyte physiology, and proved that the lymphocyte was not an end cell as had previously been thought, but could proliferate and take on new functions when stimulated properly.

Confirmation of Hungerford's and Nowell's findings was soon reported by Hastings et al. (1961), Cooper et al. (1961), Berman and Stulberg (1962), MacKinney et al. (1962) and McIntyre and Ebaugh (1962), who demonstrated that the human peripheral blood lymphocyte could be transformed in vitro into a large, morphologically primitive, "blast-like" cell capable of undergoing mitosis.

It is interesting to note that lymphocyte blast transformation had been observed and reported by Maximow and his associates as early as 1927 (Maximow et al., 1927). They stated that "the addition of tubercle bacilli to leukocytes in a plasma clot caused 'polyblastic transformation.'" Although they missed the significance of these observations at that time, they did describe the phenomenon, and were the first people to note it.
A tremendous interest was created among workers in the field of human genetics when they recognized that mitotic cells could be regularly obtained from peripheral blood, and this led to rapid development of many technical advances in the field. Low concentrations of colchicine were added to accumulate mitoses arrested in metaphase and, after Moorhead et al. (1960) had devised a fixing and spreading technique for the display of chromosomes, cytological analysis became practicable and reliable.

In spite of Hungerford's and Nowell's demonstration of the mitogenic potential of phytohemagglutinin, the investigation of the nature of the particular cell type which was stimulated was not, at first, pursued with any urgency; nor did the result of such studies seem to stir an immediate interest in lymphocyte biology. Although the responsive cell was originally thought to be a monocyte or large lymphocyte, Carstairs (1961, 1962), working at St. George's Hospital in London, was the first to clearly demonstrate that small lymphocytes were the source of the dividing cells. This was confirmed a year later by the work of Marshall and Roberts (1963a, 1963b), and by Elves and Wilkinson (1963). In several of these experiments, the initial inoculum consisted almost entirely of small lymphocytes. Once stimulated by PHA, these small lymphocytes decreased in number and, simultaneously, many blastoid cells appeared, all prior to the onset of visible cell division.

It was obvious from numerical considerations alone that transformation of many of the small lymphocytes initially present was responsible for the origin of many of the developing blastoid cells.
Lymphocyte physiology

Types of lymphocytes

Lymphocytes distinguished by size. The term "lymphocyte" is conventionally applied to a special population of cells which possess the same superficial morphology. According to Sell and Asofsky (1968), however, the division of lymphocytes by conventional morphologic criteria into large and small; or even large, medium, and small, appears to offer little advantage since the lymphocyte population is extremely heterogeneous and normal distribution into definite subpopulations has not been demonstrated. Of the population of lymphocytes, however, the small lymphocyte is by far the most numerous and has, therefore, been the most extensively studied.

As a morphological group, lymphocytes are functionally heterogeneous. It is clear from the gross morphological changes which occur on stimulation of small lymphocytes that their size, the prominence of their nucleoli, and the degree of basophilia of their cytoplasm may simply reflect the state of activity of the cells. Thus, morphologically different lymphocytes (small, medium, or large) may be a part of a functionally similar population in different states of activation.

Lymphocytes distinguished by function and origin. Within the past few years, it has become clear from numerous experimental observations that the lymphocyte is, indeed, a highly complex and active cell. The small, round cell identified by the term lymphocyte is generally an immunologically-competent, thymic-derived cell of long life span which is radiosensitive and non-phagocytic (Ling, 1968). It is an amoeboid, migratory cell which circulates through the tissues via the blood and lymph and which concent-
trates in lymphoid tissue (Gowans, 1971). Its major function is to mediate immunological reactions (Gowans and McGregor, 1965), and it is becoming apparent that different and distinct populations of lymphocytes are responsible for various components of these responses (Roitt et al., 1969).

Two distinct classes of peripheral blood lymphocytes, each capable of responding to different immunologic functions, are being widely studied at the present time (Raff, 1971). One type of lymphocyte arises as a stem cell in the bone marrow, migrates to the thymus, where it differentiates to a lymphocyte, and then proceeds out to the peripheral lymphoid tissues where it may be called a thymus-dependent, thymus-derived, thymus-processed or T lymphocyte (Raff, 1969). The other type of lymphocyte also arises as a stem cell in the bone marrow and, in birds, migrates to the bursa of Fabricius where it differentiates to a lymphocyte and then enters peripheral circulation as a bursa-derived lymphocyte (Lance and Taub, 1969). In mammals, the pathway is not clear and may simply involve the differentiation of a stem cell into a lymphocyte while still within the bone marrow; or, such differentiation may occur in peripheral lymphoid tissues, or in some gut-associated, "bursa-equivalent" tissue. Whatever the pathway, however, this type of peripheral lymphocyte can fully differentiate without having to pass through the thymus, and it has been called a thymus-independent, bone-marrow-derived, bursa-equivalent-derived, or B lymphocyte.

T and B lymphocytes, although morphologically indistinguishable at the present time, can be distinguished on the basis of surface antigenic markers: T cells are distinguished by possessing the theta (θ) alloantigen (Reif and Allen, 1964; Raff, 1969), while B cells possess either a unique complement receptor site (Lay and Nussenzweig, 1969), or (in the mouse) a
mouse-specific B lymphocyte antigen (MHIA) (Raff et al., 1971).

It has been shown experimentally that the thymus-derived T cells are mainly concerned with "cell-mediated" immune response while the thymus-independent B cells are involved with humoral-antibody-mediated immunity. Although demonstrated in many cases, this is an oversimplification (Kay, 1971) and a variety of interactions of T and B cells are now regarded as necessary for either immune response (Mills and Cooperband, 1971; Schimpl and Wecker, 1972; Wilson and Feldman, 1972).

The thymus contains two populations of lymphocytes (or thymocytes): a major one composed of short-lived cells, many or all of which appear never to leave the organ; and a long-lived population, which seems to be the source of the migrant cells (T cells) which seed the spleen and lymph nodes (Mills and Cooperband, 1971). The high rate of cell death which apparently occurs within the thymus is unexplained at the present time. Human thymocytes are immunologically incompetent in vivo (Ling, 1968) and do not respond well to phytohemagglutinin (PHA) stimulation in vitro (Claman and Brunstetter, 1968; Winkelstein and Craddock, 1967). Weber (1966), however, reported that adult swine thymus lymphocytes did respond in vitro to PHA and that responsive cells were located in the thymic medulla.

Another distinct group of cells are the marrow or myeloid lymphocytes. These cells have a short turnover time of days in the mouse (Albert et al., 1966) and may be precursors of hemopoietic cells. Whatever their function in the marrow, they cannot be replaced by lymphoid lymphocytes, and cannot substitute for them in lymphoid tissue (Cudkowicz et al., 1964).

**Lymphocyte morphology**

Morphologically, the small lymphocyte is not a striking or unusual cell.
In fixed preparations, it measures approximately 5 to 8 μ in diameter and is distinguishable from the other white blood cells because of its dark, round nucleus surrounded by a very thin rim of non-granular cytoplasm.

The nucleus, kidney-shaped and occupying most of the cell, has thick masses or clumps of dense heterochromatin lying close to the nuclear membrane. These clumps are interspersed with areas of less densely arranged euchromatin which presumably are situated near regions of nuclear "pores" (Bessis, 1961). Heterochromatin is also observed near the center of the nucleus.

Many lymphocytes have a nucleolus in the center of the nucleus, and its apparent absence in other lymphocytes, as seen in electron micrographs, may only be the result of an unfortunate plane of section (Anderson, 1966). With the light microscope, lymphocyte nucleoli are not usually seen in stained dry smears (Robbins, 1964).

As seen in electron micrographs, the cell lacks a well-developed rough endoplasmic reticulum and Golgi apparatus normally associated with protein synthesis and secretion. Relatively few mitochondria are present in the cytoplasm of the lymphocytes as compared with other cells of the body; up to a dozen round or oval mitochondria 0.4 to 0.8 μ in diameter may be grouped near the nuclear indentation or distributed around the cytoplasmic rim (Anderson, 1966; Ackerman, 1967). In appropriate sections, a centriole and a few microtubules which converge toward it are seen in the area of the nuclear indentation. The degree of cytoplasmic basophilia of lymphocytes observed in Romanovsky-stained preparations is due to the presence, relative number, and distribution of free ribosomes and polysomes scattered in the cytoplasm. The presence of cytoplasmic polysomes indicates
that the lymphocyte is actively carrying on a certain amount of protein synthesis.

**Lymphocyte function**

**Antigen recognition.** Antigen recognition is a major function of the small, circulating lymphocyte. Following *in vivo* contact with an antigen, lymphocyte proliferation serves as an amplification process, making possible an intensified response following re-exposure to the same antigen.

It is probable that the process of recognizing a foreign antigen takes place at the cell surface. The great specificity of antigen-lymphocyte reaction implies a membrane receptor similar in structure to an immunoglobulin. The term IgX has been given to such surface material found on T lymphocytes while B cells appear to have a larger number and a wider range of immunoglobulin-like recognition sites (Kay, 1971).

**Production of humoral factors.** In addition to their function in immunological surveillance, lymphocytes also serve as immunologic effector cells. When stimulated *in vivo* or *in vitro*, lymphocytes produce a great variety of humoral factors, called "lymphokines" (Dumonde et al., 1969), which serve to greatly amplify the immunologically-specific cell-mediated hypersensitivity responses.

Bloom and Bennett (1966) were the first to demonstrate convincingly that lymphocytes are the source of nonimmunoglobulin materials secreted in soluble form and possessing a specific biological activity, although suggestions that such factors might be active were present in the literature much earlier (Miller and Favour, 1951; Marks and James, 1953). Although the list appears to be growing weekly, some of these products in-
clude: migration inhibitory factor (MIF) (David et al., 1964; Bennett and Bloom, 1967; Lamelin and Vassalli, 1971), which serves to immobilize phagocytic cells (macrophages and polymorphs) entering a reaction site; inflammatory factors (Dumonde et al., 1969), which apparently exert direct cytotoxic effects on foreign or tumor cells by destroying their membranes; chemotactic factors (Ward et al., 1969, 1970), which induce migration of leukocytes to a reaction site; interferon-like factors (Wheelock, 1965; Green et al., 1969), which inhibit viral replication; mitogenic factors (Maini et al., 1969; Valentine and Lawrence, 1969; Falk et al., 1970; Spitler and Fudenberg, 1970; and Powles et al., 1971), which nonspecifically activate (recruit) those nonsensitive (normal) lymphocytes which accumulate at a reaction site, thus amplifying the overall lymphocyte response; and an inhibitor of DNA synthesis (IDS) (Smith et al., 1970; Wolstencroft et al., 1971), which may serve as a feed-back device to "control" the overall lymphocyte response.

As yet, no soluble lymphocyte product is available in a highly purified form; knowledge, therefore, of the mechanism of their production, release, and function is seriously limited. A complete discussion of these factors cannot be presented here; more detailed investigations are reported by Lawrence and Landy (1969), Dumonde (1970), and Pick and Turk (1972).

**Production of antibody.** Prior to the investigation of these non-antibody soluble mediators of lymphocyte activity, many investigators reported that specific antibody is produced by lymphocytes stimulated by PHA or antigen in vitro. Although it is well known on the basis of clinical, morphological and experimental evidence that plasma cells are the major source of antibodies and immunoglobulins (Leduc et al., 1968; La Via and
Vatter, 1969), there is some experimental evidence that circulating lymphocytes also possess these capabilities. Fluorescent staining of immunoglobulins within cultures of human lymphocytes was demonstrated after stimulation by phytohemagglutinin (Bach and Hirschhorn, 1963; Ripps and Hirschhorn, 1967), by antigens to which the cell donor was sensitized (Elves et al., 1963b; Cooperband et al., 1964), or by pokeweed mitogen (Douglas et al., 1966). Immunoglobulin synthesis has also been detected by immunoelectrophoreses or radioimmunoelectrophoresis of cell extracts or culture supernatants (Bach and Hirschhorn, 1963; Forbes, 1965; Parenti et al., 1966; Chessin et al., 1968). Jevitz and Ekstedt (1971b), working with antigen stimulated cultures, developed a micromodification of the hemolytic plaque technique of Jerne and Nordin (1963), coupled with autoradiographic observations of plaque-forming cells, and detected significant numbers of IgG-antibody-forming cells at the center of many plaques.

Several investigators, however, have failed to find immunoglobulins in transformed cells (Sell and Gell, 1965; Greaves and Roitt, 1967; Coulson and Chalmers, 1967b). McMillan et al. (1970) used a modified ammonium sulfate technique with radiolabeled human immunoglobulin fragments to detect nanogram amounts of cell-associated immunoglobulins produced by PHA-treated and nontreated lymphocytes. These investigators suggested that some immunoglobulin was synthesized and secreted by both stimulated and control cells, and that there was no enhancement observed in PHA-stimulated cultures.
Lymphocyte transformation

As living cells, lymphocytes have been observed to respond to many factors or stimuli both in vivo and in vitro. Lymphocytes respond by exhibiting changes in their metabolic and/or synthetic activities which in turn are reflected by variations in cellular morphology. At the microscopic and ultrastructural levels, these changes in lymphocyte morphology have been closely followed, providing evidence that the small cell is capable of rapid enlargement and proliferation in response to appropriate stimuli.

Morphology of blast-transformed lymphocytes

Light microscopy. The morphology of lymphoblasts, as seen by the light microscope, has been thoroughly described by Robbins (1964), Bach and Hirschhorn (1965), Elves (1966), Naspitz and Richter (1968), and Ling (1968). Within 24 hr of culture in the presence of PHA, a type of lymphocyte is detected which is larger in size than the small lymphocyte; its nucleus shows a greater amount of euchromatin, and, occasionally, a nucleolus. The cytoplasm of this cell is more extensive and more basophilic than that of the small lymphocyte. Blast cells appear between 48 and 72 hr after initial PHA stimulation. More than 70% of the small lymphocytes may be transformed into these blastoid cells. These are large cells, approximately 10 to 20 μ in size. They possess a deeply basophilic, nongranular cytoplasm surrounding a large, round nucleus which contains homogeneously-staining chromatin and one or more prominent nucleoli (Cooper et al., 1963). In the cytoplasm there is often a clear or lightly stained perinuclear zone which probably represents the Golgi apparatus. Also, there are often several small, clear cytoplasmic vacuoles (Robbins, 1964) or granules (Bach and Hirschhorn, 1965). These cells go on to divide, and numerous mitotic figures are seen between 48 and 72 hr of culture.
Many, but not all, of the blastoid cells are observed to be aggregated in dense cell clusters, a phenomenon clearly indicating where phytohemagglutinin got its name. Cooper and associates have shown that the single blast cells, as well as the free borders of cells in clusters, are actively amoeboid (Cooper, 1962; Cooper et al., 1963).

Histochemically, Cooper et al. (1963) demonstrated that glucose is utilized during blastogenesis, and lactic acid is produced at a rapid rate, suggesting that glycolysis may be the main energy source for this cellular transformation. This suggestion is further supported by Nowell's observation that blastogenesis can occur in the complete absence of oxygen (Nowell, 1960).

A characteristic morphologic feature of lymphocytes of all sizes, observed in lymphocyte preparations for decades, is a blunt cytoplasmic foot process which gives the lymphocyte a "hand mirror" appearance. This feature was described by Lewis (1931) who used time-lapse micrography, and by Rich et al. (1939), who observed that motile lymphocytes had a "tail section" so prominent that the cell could readily be distinguished from other motile leukocytes which only irregularly and briefly displayed a tail. De Bruyn (1944), who noted that lymphocyte locomotion is essentially amoeboid, suggested that the cell had "polarity", with pseudopodia being thrown out on the advancing posterior (nuclear) end of the cell, and the cytoplasmic "tail" process forming an anterior end. Termed a "uropod" (Greek: oura, tail; and podos, foot, stalk) by McFarland et al. (1966), cinephotomicrographic studies indicate that it is used by the lymphocyte to contact and attach to debris, the surface of the culture vessel, and other cells in the environment (McFarland and Heilman, 1965). Under high power
microscopy, the end of the uropod appeared to have thread-like extensions projecting outward, forming a flexible connection between the lymphocyte and other cells (McFarland, 1969).

When monolayers of other cells are present, lymphocytes are observed to crawl over them or settle neatly against them in very close contact. In this latter state, the lymphocyte appears spherical and shows little movement other than an occasional wave about its center, resembling a swirling of the cytoplasm around the nucleus (Ling, 1968). It may then move quickly and randomly for several minutes with the tail trailing behind the bulk of the cell. Occasionally, lymphocytes are observed to enter other cells and wander about inside them (Pulveraft, 1959; "emperipolesis") and, in other situations, particularly in preparations from stimulated lymphoid tissue, lymphocytes are seen to cluster around macrophages (Sharp and Burwell, 1960; "peripolesis"). Tissue culture observations thus suggest that lymphocytes associate intimately with other cells.

Lymphocytes and macrophages frequently interact in immunologic reactions in vitro. In many instances of this lymphocyte interaction with macrophages, the attachment via the uropod lasts for prolonged periods, even hours, and may be mediated by the microvilli or "microspikes" at the terminus of the uropod. Studies by Fishman and Adler (1963) and by Askonas and Rhodes (1965) have demonstrated that RNA extracted from macrophages is capable of stimulating antibody responses in vivo. The above-mentioned long-term interaction of lymphocytes and macrophages suggests that the lymphocyte may acquire stimulatory macrophage material via the uropod during the interaction. Support for such a cell-to-cell transfer of "information" was provided by Clarke et al. (1971) who used scanning electron-microscopy
to observe lymphocyte interactions with other cell types.

In an interesting ultrastructural study of uropods, Biberfeld (1971) used transmission and scanning electron microscopy to show that PHA could induce uropod formation in vitro. Biberfeld observed that after 2 days stimulation, approximately 20% of the cells had uropods, compared with approximately 5% in non-stimulated cultures. He further noted that cytotoxic action of lymphocytes directed against specific target cells was mediated in vitro by attachment of the uropod to the target cell.

Electron microscopy. Electron microscopy has contributed significantly to the study of the structure of lymphoblastoid cells. Marshall and Roberts (1963b) studied human lymphocyte response to PHA and found no evidence for an extensive endoplasmic reticulum such as is found in plasma cells. They did observe, however, numerous ribosomes and a well-developed Golgi apparatus. Marshall and Roberts noted the similarity of their electron microscopic observations to those reported by Binet and Mathe (1962) who studied the blast cell present in lymph nodes during a graft-vs-host response. Tanaka et al. (1963) noted that, after 72 hr in culture, PHA-stimulated cells had prominent nucleoli and numerous mitochondria. Inman and Cooper (1963) studied both peripheral blood and thoracic duct lymphocytes, and observed that PHA-induced blasts showed abundant mitochondria and a greatly increased cytoplasmic content of ribosomes, indicative of active protein synthesis. The highly developed Golgi apparatus was interpreted as reflecting activity associated with cell division. Elves et al. (1964) reported observations similar to the above, but claimed that the most prominent feature of the PHA-induced blast cell was the presence of large electron opaque osmiophilic bodies, membrane-bounded, which they speculated might contain globulins.
Inman and Cooper (1965) studied the relationship of cellular ultrastructure to DNA synthesis in human lymphocytes stimulated with PHA in vitro and noted that cells synthesizing DNA possessed more nucleoli, mitochondria and elaborate Golgi zones than blast cells not synthesizing DNA. Cells active in DNA synthesis possessed only small amounts of endoplasmic reticulum, but exhibited increased micropinocytosis and greater numbers of vesicles probably containing glycogen. Membrane-bounded osmiophilic bodies were also noted in some cells, as previously described by Inman and Cooper (1963), and may correspond to those bodies earlier described by Elves et al. (1964).

In an interesting study, Michalowski et al. (1966) showed that rough endoplasmic reticulum was limited in blast cells when compared to active protein-synthesizing cells. However, when lymphocytes from patients immunized with smallpox vaccine were incubated in vitro with this antigen and the blast cells obtained at the termination of culture were compared to PHA-stimulated blast cells, a more-highly-developed rough endoplasmic reticulum was observed in the antigen-stimulated blast cells.

**Transforming agents: nonspecific mitogens**

Within the past decade, many mitogenic substances have been described which can be classified as either (1) nonspecific mitogens (such as PHA) or (2) immunologically-specific "mitogens" (antigens) to which the lymphocytes being tested in vitro have been previously exposed in vivo. A partial listing of factors belonging to both of these groups is presented in Table 1.

Probably the most widely studied of either of these groups are those classified as "nonspecific." According to Oppenheim (1968), these agents are inappropriately classified as such since what is really meant by "non-
Table 1. Agents reported to cause in vitro lymphocyte blastogenesis^a

<table>
<thead>
<tr>
<th>I. Nonspecific stimulants</th>
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<tr>
<td>Phytohemagglutinin (PHA)</td>
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<tr>
<td>Pokeweed mitogen (PWM)</td>
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<tr>
<td>Concanavalin A (Con A)</td>
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<tr>
<td>Lentil mitogen (LM)</td>
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<td>Staphylococcal filtrate (SF)</td>
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<tr>
<td>Streptolysin-S (SLS)</td>
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<tr>
<td>Metal ions (mercury, magnesium, nickel, zinc)</td>
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<tr>
<td>Lymphocyte-culture supernatants (mitogenic factor)</td>
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<td>Microwave sound</td>
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<td>Antisera:</td>
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<td>anti-immunoglobulin</td>
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<td>anti-immunoglobulin allotypes</td>
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<td>anti-lymphocyte sera (ALS)</td>
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<th>II. Specific stimulants</th>
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<tr>
<td>Tissue antigens:</td>
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<tr>
<td>homologous lymphocytes</td>
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<td>heterologous lymphocytes</td>
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<tr>
<td>extracts of white blood cells</td>
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<td>Tuberculin (purified protein derivative - PPD)</td>
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<tr>
<td>Histoplasmin</td>
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<tr>
<td>Tetanus toxoid</td>
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<tr>
<td>Typhoid-paratyphoid vaccine</td>
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<tr>
<td>Diphtheria toxoid</td>
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<tr>
<td>Polio vaccine</td>
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<tr>
<td>Vaccinia (smallpox) vaccine</td>
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<tr>
<td>Streptolysin-0</td>
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<tr>
<td>Ragweed pollen</td>
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<tr>
<td>Penicillin</td>
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<tr>
<td>Keyhole limpet hemocyanin</td>
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<tr>
<td>Horse ferritin</td>
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<tr>
<td>Red blood cells</td>
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<td>Antigen-antibody complexes</td>
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</table>

^a Modified from Oppenheim (1968) and Hirschhorn (1969)
specific" is that the mechanism of action is unknown. None of these stimu-
lants need prior sensitization of the cell donor to be effective, and all of
them are capable of stimulating human cord blood lymphocytes. PHA is the
most widely studied agent in this group and so will be discussed first.

Phytohemagglutinin. Much of the foregoing review has described the
observations of PHA-induced blastogenesis. Originally, however, phytohemag-
glutinin was used to agglutinate red blood cells in a whole blood suspension,
as first discovered and described by Landsteiner and Raubitschek (1908), and
much later by Li and Osgood (1949). It was for erythroagglutination that
Hungerford et al., (1959) and Nowell (1960) had been using PHA when they
discovered its mitogenic potential. According to Naspitz and Richter (1968),
PHA will adequately agglutinate the erythrocytes of human, rabbit, dog, cat,
chicken, duck, mouse, rat, sheep, horse, pig, frog, and guinea pig, but will
only weakly agglutinate bovine erythrocytes.

Phytohemagglutinin is a saline extract from red kidney beans, Phaseolus
vulgaris, and, as prepared by the method of Rigas and Osgood (1955), has
come to be known as PHA-M (PHA-mucoprotein). According to Naspitz and
Richter (1968), this mucoprotein was very soluble in water and behaved as
a homogeneous substance by electrophoretic analysis over the pH range of
5.8 to 8.6. Below pH 5.8 it dissociated into a protein phytohemagglutinin
and an inactive polysaccharide. This protein phytohemagglutinin (PHA-P)
behaved as a homogeneous substance by electrophoresis over the pH range of
2.0 to 8.0, with an isoelectric point of 6.5. PHA-P was also shown to be
a heat-labile euglobulin, insoluble in distilled water, but very soluble
in saline or buffer solutions.

Rigas and Johnson (1964) found PHA-P to be a homogeneous protein made
up of 8 subunits, with an approximate molecular weight of 128,000. They showed that the hemagglutinating and mitogenic activities were to be found on separate subunits and could therefore be separated.

In addition to separate hemagglutinating and mitogenic potentials, PHA at higher concentrations also has the property of agglutinating leukocytes in vitro (Kolodny and Hirschhorn, 1964; Naspitz and Richter, 1968). Leukoagglutinating activity and mitogenic activity are apparently closely linked, since hemagglutinating activity of PHA could be removed by repeated absorption with red cells without impairing the leukoagglutinating and mitogenic activity. However, if leukoagglutinating activity was removed by repeated white cell absorption, mitogenic and hemagglutinating activity were also lost (Rivera and Mueller, 1966; Weber et al., 1967; Richter and Naspitz, 1967).

**In vitro activity of PHA.** Approximately 60 to 90% of normal lymphocytes cultured for several days in the presence of PHA will develop into blast-like cells. Astaldi and Airo (1967) cultured human blood lymphocytes at 2 x 10^6 cells/ml in medium containing 20% autologous plasma. After 3 to 4 days in the presence of PHA, they observed that from 60 to 80% of the lymphocytes had become blast-like cells. These results were supported by Ling (1968) who reported 70 to 90% of human lymphocytes were transformed by PHA. Sell et al. (1965) reported 80% blast forms in rabbit lymphocyte cultures at 48 hr after PHA stimulation.

Muñiz et al. (1970) stimulated human lymphocytes in vitro with 0.02 ml of reconstituted PHA-P solution/10 ml of culture. Cultures were prepared with 6 x 10^6 cells in 10 ml of medium containing 15% fetal calf serum. Maximum lymphocyte transformation, as determined by nuclear diameter, occurred
at approximately 72 hr after stimulation. Smith et al. (1971) also used
PHA-P to study normal human lymphocyte metabolism and blastogenesis in cul-
tures which contained 20% fetal calf serum. Reconstituted PHA-P was diluted
1:5 in Gey's balanced salt solution and 0.05 ml of this was added to 1.5 ml
of cell suspension prepared at an optimal cell density of $10^6$ cells/ml of
culture. Horký (1970) used PHA-P at a final culture concentration of 0.02%
in cultures of human lymphocytes set up at a cell concentration of $0.8 \times 10^6$
cells/ml of culture. Maximum blastogenic response was found in cultures ter-
minated after 72 hr incubation. In an ultrastructural study of blast-cell
morphology, Biberfeld (1971) cultured human lymphocytes at $10^6$ cells/ml of
Eagle's medium which contained 5% fetal calf serum. Cultures were examined
after 2 days incubation with PHA-P at a final culture concentration of 40
µg PHA-P/ml of medium. Sample and Chretien (1971) used PHA-P at a culture
level of 6.25 µg/ml of culture. Human lymphocytes were set up at a density
of $0.5 \times 10^6$ cells/ml of culture which contained 20% serum. Cultures were
terminated at times ranging between 48 and 72 hr.

McMillan et al. (1970) studied human lymphocyte response to PHA-M.
They used 0.25 ml of PHA-M per culture to achieve maximum lymphocyte stimu-
lation. Cultures consisted of 10 to $20 \times 10^6$ cells in a final volume of
10 ml of TC-199 medium.

Dowd et al. (1964) cultured lymphocytes from heparinized rat blood in
tissue culture with PHA-M (0.1 ml PHA-M in 16 ml of culture medium). Good
growth was obtained in the cell concentration range of from 5 to $30 \times 10^6$
cells/ml with an optimum at $10 \times 10^6$ lymphocytes/ml. Fetal calf serum was
not as good as newborn agammaglobulin calf serum in permitting stimulated
mitotic activity. Rieke and Schwarz (1964) also worked with heparinized
rat blood lymphocytes. PHA-P was used as mitogen (0.01 ml of a 1:5 dilution of stock PHA-P/ml of blood). Cultures were set up at from 1 to 5 x 10^6 cells/ml of Eagle's medium. Twenty-percent fresh rat serum was used. The use of fresh, rather than frozen serum was considered important.

The response of guinea pig lymphocytes to stimulation by PHA was investigated by Zweiman and Phillips (1970). They found that cells in cultures which contained 10^6 cells/ml responded to different levels of PHA-P with a dose-dependent response. Maximum culture activity occurred at 0.5 to 1.0 µl PHA-P/ml of culture. Higher concentrations than this resulted in decreased isotope incorporation. Maximum isotope incorporation occurred on the third day of culture.

Fernald and Metzgar (1971) used a semi-micro method to study the in vitro blastogenesis of hamster lymphocytes. They used 10 µl PHA-P per culture to achieve optimal results. Cells were grown in 15% autologous or pooled hamster serum at from 0.5 to 1 x 10^6 cells in a 0.2-ml culture volume.

Many factors have been described (in addition to PHA) which nonspecifically induce blastogenesis in the majority of cells in a given lymphocyte suspension.

Pokeweed mitogen. An extract from the pokeweed plant Phytolacca americana (pokeweed mitogen, PWM), has been shown to be a potent lymphocyte activator (Farnes et al., 1964; Barker et al., 1965; Börjeson et al., 1966; Chessin et al., 1966). Barker (1969) presented electron microscopic evidence that small lymphocytes are capable of complete cytologic and ultrastructural differentiation to plasma cells in an in vitro environment in response to pokeweed mitogen, a phenomenon never observed in PHA-stimulated
cultures. According to Chessin et al. (1966), the PWM-stimulated lymphocyte developed into an intermediate-sized cell type by 72 hr and contained an eccentric nucleus, clumped heterochromatin, a well-developed Golgi apparatus, ribosomal aggregates, and a well-developed rough-surfaced endoplasmic reticulum.

Concanavalin A. Another bean extract which shows definite mitogenic activity is Concanavalin A (Con A), an extract from the Jack bean, Canavalia ensiformis (Powell and Leon, 1970; Novogrodsky and Katchalski, 1971; Janossy and Greaves, 1972). The binding of Con A to lymphocytes can be reversed at any time during blastogenesis simply by adding to the culture medium sugars which preferentially bind Con A (Goldstein et al., 1965).

Lentil mitogen. A potent lymphocyte activator, lentil mitogen (LM), has been extracted from Lens culinaris (Hashem and Kabarity, 1966; Young et al., 1971; Janossy and Greaves, 1972). LM has been reported to be as effective a mitogenic agent as PHA, capable of inducing from 60 to 89 percent of the lymphocytes in a cell suspension into blast formation.

Additional plant extracts with some mitogenic activity were reported by Barker (1969) and Hashem and Kabarity (1966), and include extracts from broad beans (Vicia faba) and fenugreek (Trigonella faenum grsecum).

Bacterial products. Other nonspecific mitogens include several metabolic products obtained from the growth medium of bacterial cultures; they include staphylococcal filtrate (Ling and Husband, 1964; Ling et al., 1965; Sell et al., 1965; Peavy et al., 1970) which consists of a filtered supernatant of cultured staphylococci; streptolysin-S (SLS) (Hirschhorn et al., 1964) which is a hemolytic exoprotein produced by streptococcal organisms; and a Salmonella endotoxin (Peavy et al., 1970).
Metal ions. Nonspecific lymphocyte stimulation has been reported for several heavy-metal ions, such as mercuric chloride (Schöpf et al., 1967; Caron et al., 1970); nickel acetate (Pappas et al., 1970); magnesium chloride (Perris et al., 1967; Whitfield et al., 1969), and zinc ions (Kirchner and Rühl, 1970).

Events following mitogenic stimulation of lymphocytes

Response of T and B cells to mitogens. Allan et al. (1971) suggest that the initial event in transformation of lymphocytes by PHA is the selective binding of the PHA mitogen by a receptor on the cell surface. Borberg et al. (1966) suggested that N-acetyl-D-galactosamine (NAGA) is an important constituent of this PHA-combining site on the cell. They found that NAGA inhibited the agglutination of rat thoracic duct cells by PHA while other sugars such as D-glucose, N-acetyl-D-glucosamine, D-galactose and D-mannose had little or no effect.

Greaves et al. (1972) reported that virtually 100% of all T and B lymphocytes have binding sites for the three phytomitogens PHA, LM, and Con A. In an earlier study, Janossy and Greaves (1971) investigated the selective activation of T and B lymphocytes by the various mitogens and clearly demonstrated that the responsiveness to PHA is predominantly, if not uniquely, a property of T lymphocytes, in spite of the presence of receptors on B cells. In contrast to PHA, however, pokeweed mitogen (PWM) is found to activate mostly B cells in vitro. One of the most interesting aspects of this lymphocyte blastogenic response to PWM is that a considerable proportion of the activated cells develop ultrastructural features characteristic of plasma cells, a response also considered to be characteristic of B cells.
in vivo (Barker, 1969). Recently, Janossy and Greaves (1972) reported that LM and Con A activate T cells only, and not B lymphocytes.

**Mechanism of mitogenic activation.** Most investigators who have worked with "nonspecific" lymphocyte mitogens have at one time or another speculated as to the mechanism of such cellular activation. Nowell (1960) theorized that PHA might alter the cell membrane of the lymphocyte so that some substance in the culture medium could enter the cell and initiate the mitotic process. Pearmain et al. (1963) suggested a specific immunological response to PHA, acting as an ubiquitous antigen. An alternative view was proposed by Hirschhorn et al. (1963) who theorized that a nonspecific immunological response to PHA occurred, and demonstrated that PHA stimulated γ-globulin production. Coulson and Chalmers (1964b), suggested that, instead of playing the role of an antigen, PHA acted as a drug, nonspecifically stimulating transformation and mitoses and even γ-globulin production. Jackson and Killander (1964) proposed that PHA acted primarily to stimulate active cell growth, which would normally include DNA synthesis, rather than by direct stimulation of mitosis. Yoffey et al. (1965) supported this point of view.

Hastings et al. (1961) observed that mitoses occurred only in white blood cell clumps and thus felt that leukoagglutination was partially responsible for the mitotic activity. Kolodny and Hirschhorn (1964) could not separate the leukoagglutinating and mitogenic activities in purified PHA, although both functions could be separated from hemagglutinating activity by repeated red cell absorption. They also concluded that there was an apparent relationship between mitotic activity and white blood cell clumping. Hülser and Peters (1971) and Yachnin et al. (1972) have recently
supported this view with evidence that cell-cell interaction potentiates
the action of phytohemagglutinin.

Rivera and Mueller (1966) demonstrated that the mitogenic and leuko-
agglutinating factors in PHA were separable and structurally independent
of each other. They then proposed that PHA functions by independently
stimulating RNA and DNA synthesis. Hirschhorn et al. (1969) suggested that
PHA acts by increasing the rate of RNA transcription both at previously-
accessible and newly-released template sites on the DNA molecule, thereby
increasing the RNA production of the cell. This view has also received
support from Cooper (1970). Rubin et al. (1970) theorized that a cell sur-
face mechanism might be rapidly activated by the interaction of PHA mole-
cules with receptor sites on the cell membrane. They observed that minutes
after exposure of lymphocytes to PHA, certain acid hydrolases shifted from
particulate form (lysozomes) to the cytoplasmic supernatant, and that,
during the following 24 to 48 hr, PHA-treated lymphocytes developed many
acid hydrolase-rich granules. This suggested that PHA acted by causing the
release of lysosomal-bound hydrolytic enzymes. Rubin et al. (1970) theo-
rized that the enzymes then passed from the cytoplasm to the nucleus of the
cell where they removed proteinaceous repressor materials (histones) from
DNA, causing subsequent widespread gene activation.

Fanger et al. (1970) have observed that, whatever the exact mechanism
of stimulation may be, it is markedly enhanced in the presence of the re-
ducing agents L-cysteine, glutathione, or sulfite. On the other hand, a
large number of substances have been shown to depress or suppress lymphocyte
transformation, such as DEAE-dextran (Rigby, 1969); aflatoxin-B₁ (Savel et
al., 1970); L-asparaginase (Miura et al., 1970); and L-glutaminase (Hersh,
1971). Also, there are a number of diseases in which there is a defective \textit{in vitro} response of lymphocytes which may be correlated with impaired \textit{in vivo} delayed hypersensitivity and/or decreased serum immunoglobulin levels, such as in miliary and active tuberculosis, leprosy, sarcoidosis, Hodgkin's disease, and chronic lymphocytic leukemia (Oppenheim, 1968). A full discussion of those situations and diseases which lead to impaired \textit{in vitro} lymphocyte transformation is presented in reviews by Oppenheim, 1968; Rubin \textit{et al}., 1970; and Mills and Cooperband, 1971.

\textbf{Sequence of mitogen-induced biochemical events.} The sequence of biochemical events which occurs following addition of PHA to lymphocyte cultures has received much study, and the dynamics of the lymphocyte response to PHA is well established. Broadly, the sequence is an early increase in the rate of RNA synthesis followed by an acceleration in the rate of DNA synthesis (Epstein and Stohlman, 1964), presumably reflecting the process of chromosomal replication prior to mitosis. Mueller and Mahieu (1966) reported a sequence of RNA synthesis followed by protein synthesis and DNA synthesis. Sell \textit{et al}., (1965) found that after \textit{in vitro} stimulation of rabbit peripheral lymphocytes with PHA, the sequence of events was: protein synthesis, RNA synthesis, histologic blast transformation, DNA synthesis, and then mitosis, with the complete sequence requiring 48 hr.

McIntyre and Ebaugh (1962) observed that RNA synthesis began within the first 24 hr after exposure to PHA. Cooper and Rubin (1965) showed that the earliest change following PHA stimulation was a decrease in total cellular RNA, detectable after 30 min exposure to the mitogen. After 1 hr, accelerated synthesis of RNA, shown by incorporation of tritiated-uridine, was detected, and a progressive increase in the rate of synthesis continued
for 24 hr. Cooper and Rubin (1966) showed that most of this rapidly synthesized RNA was not associated with ribosomes. Mueller and Mahieu (1966) reported that RNA production increased exponentially as soon as PHA was added to cell cultures and that, by centrifugal analysis of the newly synthesized RNA, all classes of RNA were stimulated by PHA.

DNA synthesis begins 24 to 48 hr after the addition of PHA (Kay, 1967; Robbins, 1964). This conclusion is based on studies involving the incorporation of radiolabeled pyrimidine nucleosides like tritiated-thymidine (Cooper et al., 1961; Bender and Prescott, 1962; MacKinney et al., 1962; Michalowski, 1963; Aisenberg, 1965), and such non-specific labels as radioactive phosphorus (P\textsuperscript{32}) (Ling and Husband, 1964; McIntyre and Ebaugh, 1962) into newly synthesized DNA. Yoffey et al. (1965) investigated human lymphocyte transformation by means of autoradiography and liquid scintillation analysis of \textsuperscript{3}H-thymidine labelling of stimulated cells. They described in detail the morphological alterations and nuclear DNA labelling which occurred after PHA stimulation. Hartog et al. (1967) made similar studies on tuberculin PPD-stimulated cells and confirmed the validity of measuring DNA synthesis in stimulated lymphocytes by uptake of radioactive thymidine.

The incorporation of labeled precursors into DNA, RNA, or protein molecules has been a useful tool in the study of cell physiology (Cooper et al., 1961). However, according to Smets (1969) and Sample and Chretien (1971), the basic assumption that the amount of incorporated precursor is proportional to the amount of neo-synthesized molecules may not always be valid. Since radioactive precursors enter intracellular pools, fluctuation in these pools can markedly stimulate or reduce incorporation rates without corresponding changes in the actual rate of DNA, RNA, or protein synthesis.
About the only way to avoid this "pool" problem is to investigate radiolabeling in cell-free filtrates, as did Hausen et al. (1969). Further, Kay and Handmaker (1970) noted that there is no simple relationship between uridine incorporation into RNA and the rate of RNA synthesis, since the former is limited by the rate of phosphorylation of uridine by uridine kinase.

Transforming agents: specific lymphocyte stimulants

The discovery that PHA could induce a similar blastogenesis in nearly all normal lymphocytes suggested to many investigators that it might have an immunologic basis for its action, prompting a wide search for other factors which might have similar activity.

Tissue antigens. Before discussing the "specific" stimulants of lymphocyte blastogenesis, it is of interest to note that a number of tissue antigens are capable of stimulating lymphocytes in vitro. This was first reported by Bain et al. (1964) who observed that mixed leukocyte cultures (MLC) of genetically unrelated individuals showed a moderate degree of lymphocyte transformation. They found that no prior sensitization of either of the cell donors to the antigens of the other was necessary, and that the degree of reaction was related to the genetic disparity of the two cell types tested. Also described by Bach and Hirschhorn (1964) and Rubin et al. (1964), it is thus a two-way reaction in which the formation of "blast" cells is interpreted as a response of lymphocytes of one person to the foreign histocompatibility antigens on the cells of the second individual (Moynihan et al., 1965). Generally speaking, live cells are usually much more effective as stimulators than are leukocyte extracts or
metabolic products (Hashem and Rosen, 1964; Oppenheim, 1968). These observations have led to a tremendous literature on the subject of mixed lymphocyte reactions. A detailed discussion of the subject is presented by Ling (1968).

**Immunoglobulin molecules.** In addition, a wide variety of antisera prepared against white cells (Grasbeck et al., 1963; Knight and Ling, 1967) and against various immunoglobulins or immunoglobulin allotypes (Gell and Sell, 1965; Sell, 1967, 1968) have also been demonstrated to be very effective stimulators of *in vitro* lymphocyte proliferation. According to Sell and Gell (1965), even small genetic dissimilarities between different strains of rabbits can be demonstrated in this way.

**Soluble antigens.** Probably the largest group of lymphocyte activators are the specific antigenic stimulants of lymphocytes taken from hosts previously sensitized to those antigens. A complete list of such agents, many of which commonly cause delayed hypersensitivity reactions, would be so large as to include most of those antigens which have been used over the years to sensitize experimental subjects. A partial listing of some of these antigens is shown in Table 1, page 24.

Tuberculin (purified protein derivative, PPD) is the prototype and most thoroughly studied of the specific antigens in this group. It was, in fact, the first known blastogenic factor, other than PHA, to be studied. In a historic short paper from New Zealand, Pearmain et al. (1963) reported that blast cells and mitoses developed in lymphocyte suspensions from the blood of donors who had been infected with tuberculosis, or who had been immunized with tubercle bacillus, when such suspensions were cultured in the presence of PPD, whereas lymphocytes from tuberculin negative
patients, cultured under the same conditions, showed no blast cells. Simultaneously and independently, similar results were reported by Schrek (1963), thus providing mutual confirmation of this in vitro response to specific antigen. (It is interesting to note that this concept of antigenic stimulation of lymphocytes in vitro at one time held no credence. Pearmain's original report to The Lancet was almost rejected on the grounds that the results were impossible!) Soon recognized, however, as evidence that peripheral lymphocytes from sensitized donors could respond in vitro with a secondary immunospecific response to an antigen, these early reports were confirmed by others (Marshall and Roberts, 1963a; Cowling et al., 1963; Aspegren and Rorsman, 1964a,b).

Specific antigenic agents generally transform from 5 to 40% of peripheral human lymphocytes, in contrast to the 70 to 90% transformation when stimulated by PHA, provided that the cells have been obtained from previously sensitized subjects (Oppenheim, 1968). There is evidence that this response in vitro parallels and has several characteristics of delayed hypersensitivity in vivo (Mills, 1966; Zweiman, 1967; Ling, 1968; Jevitz and Ekstedt, 1971a). Zweiman and Phillips (1970) have shown that the in vivo hypersensitivity response to tuberculin in guinea pigs could be completely suppressed by β-mercaptoethanol treatment without affecting the ability of sensitized lymphocytes from such animals to respond to antigen in vitro.

Soon after the action of tuberculin was reported, Elves et al. (1963a) reported studies with a variety of antigens and found that greater than 30% of cells in stimulated cultures responded with blast formation. Antigens stimulating these secondary responses included tetanus toxoid, typhoid-
paratyphoid vaccine, diphtheria toxoid, poliovirus vaccine, and smallpox vaccine. The blastogenic response was observed as early as 72 hr after stimulation. Lycette and Pearmain (1963) reported significant in vitro stimulation of sensitized cells by Sabin poliovaccine and ragweed pollen. Hirschhorn et al. (1964) found Streptolysin-O and keyhole limpet hemocyanin were also active in vitro. With regard to penicillin sensitivity, the lymphocyte blastogenic response to this antigen was reported to occur in subjects with immediate wheal reactions as well as in those with delayed skin reactions, suggesting that lymphocyte transformation might be associated not only with delayed hypersensitivity, but also with immediate, Arthus-type reactivity (Pearmain et al., 1963; Hirschhorn et al., 1963).

In vitro antigenic stimulation by a variety of albumins and serum globulins was reported by Dutton and Eady (1964) and by Mills (1966).

When studying tuberculin activation of lymphocytes, an investigator can choose to use either dilutions of Koch's "Old Tuberculin" (OT) preparations, or the purified protein derivative, PPD (Ling, 1968). Schrek (1963) used PPD at a final concentration of 5 to 20 µg/ml and observed blast transformation of sensitive cells after 3 to 5 days of culture. Aspegren and Rorsman (1964a) found that OT at a concentration of 1/2,000 induced mitoses in 70% of the 6-day cultures prepared from tuberculin-positive persons, while cultures prepared from tuberculin-negative persons showed very few mitoses at this concentration of antigen. Also using OT, Cowling et al. (1963) found that stimulated cultures exhibited a dose response; i.e., cultures did not respond as well at low or high concentrations of antigen as they did at intermediate antigen levels. They showed that the degree of transformation was fairly constant in the range of
1/250 to 1/2,500 dilution of OT with tailing off at high and low concentrations. Thus, although blast transformation occurs more slowly in antigen-stimulated than in PHA-stimulated cultures, the transformation is apparently less affected by changes in stimulant concentrations.

With a seven day culture period and $10^6$ cells/ml, Caron (1966) found maximal activity in cultures containing 1.25 µg PPD/ml of culture, at least one-fourth the concentration reported by Schrek (1963). Coulson and Chalmers (1967a) found that a PPD concentration of 250 OT units/ml of culture containing $2 \times 10^6$ cells/ml gave the best response when measured at the most satisfactory culture period of 90 hr. Hartog et al. (1967) found that $2.5 \times 10^{-2}$ mg PPD/culture gave maximum stimulation of cultures in a typical dose response curve, and the maximum uptake of tritiated thymidine occurred on the 6th day after stimulation. Oppenheim (1968) used 10 µg PPD/ml of cultured guinea pig cells to achieve optimal stimulation. Valentine and Lawrence (1969) worked with as little as 0.6 µg PPD/ml. In a thorough study of the in vitro response of guinea pig blood lymphocytes to both phytohemagglutinin and tuberculin, Phillips and Zweiman (1970) used Koch's OT to stimulate cell cultures consisting of $10^6$ cells/ml. They observed specific in vitro blast transformation of guinea pig cells to tuberculin antigen, in contrast to the results of Aspegren and Rorsman (1964b) and Marshall and Roberts (1963a), who were unable to do so. Like Cowling et al. (1963), Phillips and Zweiman observed a "dose-response" of cultured cells to varied concentrations of antigen. They found that the best tuberculin concentration in their system was in the range of from 0.25 to 2.5 mg OT/ml of culture. Cells from unsensitized animals did not respond to the antigen, while maximum isotope incorporation by lymphocytes from sensitized
animals occurred on the 5th and 6th day of culture. Fernald and Metzgar (1971) reported that hamster lymphocytes from immunized animals responded maximally on day 5 after stimulation with from 5 to 10 µg PPD/ml.

Mills (1966) suggested that a large proportion of both the long- and short-lived populations of sensitized lymphocytes underwent blast cell transformation in vitro. More recently, however, Marshall et al. (1969) used time-lapse cinematography to show that most of the blast cells which appeared in tuberculin-stimulated cultures on days 5 to 7 were derived by repeated mitosis from about 2% of the starting population. They suggested that recruitment of lymphocytes not themselves initially sensitive to antigen might also serve (especially during the first 2 days of culture) as an additional mechanism to enlarge this hypersensitive response of lymphocytes. That this type of recruitment does occur was clearly demonstrated by Valentine and Lawrence (1969), who reported that tuberculin-sensitive human lymphocytes cultured for 36 hr with tuberculin PPD elaborated a soluble material which caused nonsensitive lymphocytes to respond to PPD in vitro with transformation and proliferation. The elaboration of such a mitogenic factor is not out of keeping with the great variety of biologically-active substances ("lymphokines") known to be synthesized in vitro by PHA- or antigen-stimulated lymphocytes (Lawrence and Landy, 1969).

Experimental work with bovine lymphocytes

Although a great variety of experimental animals have been tested for in vitro lymphocyte activity, only limited work has been done with bovine lymphocytes, due in large part, no doubt, to previous problems in obtaining these cells from peripheral blood.
There are many techniques available for separating peripheral blood lymphocytes from granulocytes and erythrocytes, most of which depend on differences in the phagocytic and adhesive properties of the cells. In spite of the relative ease with which lymphocytes of many species may be separated on glass beads and glass wool (Wildy and Ridley, 1958; Johnson and Garvin, 1959; Rabinowitz, 1964), nylon wool (Greenwalt et al., 1962), or by differential sedimentation (Coulson and Chalmers, 1964a; Sell and Gell, 1965; Hulliger and Blazkovec, 1967), the use of similar methods to prepare purified suspensions of bovine lymphocytes has, for unknown reasons, met with singular lack of success. Burrin et al. (1966) reported a very cumbersome technique for isolating bovine blood lymphocytes that took from 24 to 36 hr to complete. Joel et al. (1969) described a technique for separating small numbers of lymphocytes from the blood of calves and goats, using a modification of an earlier procedure using silicone fluids, reported by Carper (1967). Larkin and Lin (1971) reported large scale separation of lymphocytes from bovine blood using a very specialized apparatus called a hypotonic-gyrotester. However, the viability of isolated cells was reported to be as low as 16%. Needing large quantities of viable bovine lymphocytes requiring a minimal amount of time and equipment to isolate, Kay and Kaeberle (1972) developed a simplified cotton-fiber column for separating bovine lymphocytes from the buffy-coat cell layer of defibrinated blood (see the description of this procedure in the Materials and Methods section to follow). Cotton wool columns have been used to separate leukocytes (Fichtelius, 1951; Walker et al., 1961; Cooper and Rubin, 1965), but have the problem of slow flow rates, large size and considerable loss of cells in the packing material.
There are very few published reports of studies involving normal bovine lymphocytes in cell culture. Biggers and McFeely (1963) and Ulbrich et al. (1963) reported methods to study bovine chromosome structure, and used the white blood cells obtained from the buffy coat of bovine blood. Hausen et al. (1969) reported a study of RNA synthesis in PHA-stimulated bovine lymph node lymphocytes. This was investigated by three independent methods: (1) by measuring net RNA synthesis in a culture; (2) by pulse-labeling with $^{14}$C-uridine; and (3) by determining the RNA synthesizing capacity of cell free systems. The cells studied were taken from the nodes of freshly slaughtered 2-year old animals, filtered through a glass wool column, distributed at a concentration of $5 \times 10^6$ cells/ml in 250-ml culture volumes, and preincubated in vitro for 15 hr before stimulation. Test cells were homogenized and assayed for soluble and DNA-bound RNA polymerase activity after a 36-hr incubation with PHA.

Aalund et al. (1970), in studies of bovine leukocytes obtained from whole buffy-coat suspensions, set up a leukocyte migration inhibition test to detect in vitro delayed hypersensitivity to Mycobacterium paratuberculosis. Preliminary results indicated that such a test might be useful for pre-clinical diagnosis of M. paratuberculosis infection.

Hülser and Peters (1971) investigated the in vitro transformation of bovine lymph node lymphocytes, as had Hausen et al. (1969), and concluded that cell-cell contact in PHA-clumped cell aggregates enhanced the blastogenic response of tested cells. By means of glass microelectrodes, they demonstrated that there was a flow of ions between cells which were in contact, and postulated that this type of contact might be a form of intercellular communication. These investigators found that the normally
high ohmic resistance of cell membranes was reduced at the sites of PHA-induced cell contact. They also reported that, in stimulated lymphocyte cultures, the rate of uridine incorporation was in fact a function of the degree of cellular agglutination.
MATERIALS AND METHODS

Preparation of cells and cultures

Glassware

Lymphocyte cultures were set up in 16 x 125 mm "Ready to Use" (RTU) disposable glass culture tubes\(^1\). The tubes were sterilized by dry heat and were individually capped with loose-fitting plastic "Kap-uts"\(^2\) which had been sterilized by autoclaving. Fifty-ml screw-capped tubes (25 x 150 mm) were routinely used for centrifugation steps used in isolating and washing bovine lymphocytes.

Bovine blood was collected in 1,000-ml Erlenmeyer flasks which contained nine 6-inch wooden applicator sticks for defibrinating the blood. The sticks were attached to each other by a tightly-wound rubber band to form a vertical "cone" which did not rotate with the blood (Figure 1, page 49).

Before use, all glassware items used in the collection and isolation of bovine lymphocytes were thoroughly cleaned by washing in Haemo-Sol cleanser\(^3\), after which they were rinsed 15 times with tap water, 10 times with distilled water, and once with double-distilled de-ionized water.

All glassware used in handling radioactive culture fluids and waste liquids were placed after use in plastic radioactive-waste disposal bags, while the radioactive waste liquids were stored in large glass bottles.

\(^1\)Becton, Dickinson and Company, Rutherford, New Jersey
\(^2\)Bellco Glass Inc., Vineland, New Jersey
\(^3\)Meinecke and Company, Baltimore, Maryland
Both were picked up for disposal by the Radiological Services Group, Iowa State University.

Media

All culture media used in this study were purchased from Grand Island Biological Company, Grand Island, New York, and were stored at 4°C until used, except that antibiotics, L-glutamine, and serum were stored at -20°C. Those media and solutions which were purchased in a concentrated form were diluted to proper strength with double-distilled de-ionized water.

As a routine means of controlling any contaminating microbial growth, antibiotics were added to all culture media and salt solutions prior to their use. The antibiotic stock solution (100X) consisted of the following: penicillin, 10,000 units/ml; streptomycin, 10,000 mcg/ml; and Fungizone, 25 mcg/ml.

Eagle's Minimum Essential Medium (MEM), with Earle's salt base, was used as the standard medium for the in vitro culture of bovine peripheral blood lymphocytes. To this medium was added antibiotic stock solution (10 ml/liter), sodium bicarbonate for pH control (2.25 g/liter), L-glutamine (292 mg/liter), and newborn calf serum⁴ to a final concentration of 20% (v/v).

Eagle's Minimum Essential Medium without leucine (MEM-leu), with Earle's salt base, was used when the study of lymphocyte protein synthesis required that ¹⁴C-labeled leucine be used as a tracer in the cultured cell suspensions. L-glutamine (292 mg/liter), antibiotic stock solution (10 ml/
liter), and newborn calf serum (20%, v/v) were also added to this medium.

Earle's Balanced Salt Solution (EBSS), calcium and magnesium free, was prepared with added antibiotic stock solution (10 ml/liter) and sodium bicarbonate (750 mg/liter) and was the solution used to wash and suspend the lymphocytes during their isolation and purification.

Radioisotopes

Three different radiolabeled materials were used in the in vitro studies of bovine lymphocytes. Tritiated thymidine ($^3$H-T; specific activity 6.7 Ci/mmol)$^5$ was used in the studies of DNA synthesis; tritiated uridine ($^3$H-U; specific activity 14 Ci/mmol)$^5$ was added to cultures for incorporation into newly synthesized RNA; carbon-14-leucine ($^{14}$C-leu; specific activity of 262 mCi/mmole)$^5$ was used for study of protein synthesis.

Source of lymphocytes

The blood lymphocytes used in this study were taken at different times from 21 head of cattle, including test and control animals. The animals were chosen without regard to their sex, age, or breed from a herd housed in an open shed at the Veterinary Medical Research Institute, Iowa State University. No special tests for animal health were made, aside from the observance of healthy appearance as shown by apparent good appetite and lack of outward signs of clinical disease or stress. All sensitized animals had been made sensitive to tuberculin by means of two subcutaneous injections with an inoculum containing 5 ml of complete Freund's adjuvant$^6$

$^5$New England Nuclear Company, Boston, Massachusetts

$^6$Bacto Adjuvant Complete, Difco Laboratories, Detroit, Michigan
(containing 1 mg/ml *Mycobacterium tuberculosis* strain H37 Ra). Three head of cattle housed at the Veterinary Obstetrics Barn, Iowa State University, and which had no known previous exposure to *M. tuberculosis*, were used as donors of control lymphocytes.

**Collection of bovine blood**

Blood was aseptically withdrawn from the jugular vein of test animals and collected by vacuum into sterile Erlenmeyer flasks (Figure 1, page 49). Up to 1,000 ml of venous blood were collected from each animal and defibrinated by rapidly swirling. Defibrination was complete after 5 to 10 min of swirling, or when large fibrin clots were evident on the sticks.

**Isolation of lymphocytes**

The defibrinated blood was aseptically transferred to 50-ml tubes and centrifuged for 40 min at 750 x G in a swinging-bucket-type centrifuge\(^7\) to separate cells and serum. The thin, white, buffy-coat cell layer containing lymphocytes and granulocytes, which formed at the erythrocyte-serum interface, was collected with a pipette. Care was taken to remove as few of the underlying red cells as possible.

The lymphocytes and remaining granulocytes were separated by taking advantage of the adhering tendencies of the latter. A small column of short cotton threads was made by partially filling a 35-ml disposable plastic syringe\(^8\) with fibers pulled from one-inch cut squares of cotton gauze ("cheesecloth") (Kay and Kaeberle, 1972). A short length of 1/8-inch rubber

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\(^7\)International Centrifuge, Model UV; International Equipment Co., Boston, Massachusetts

\(^8\)Monoject; Sherwood Medical Industries, Inc., Deland, Florida
Figure 1. A photograph of the type of bleeding apparatus used in the collection and defibrination of bovine blood. A 1,000-ml Erlenmeyer flask was fitted with a rubber stopper containing a blood-inlet hose and a vacuum hose outlet. A vertical "cone" of nine 6-inch applicator sticks, bound together near one end with a rubber band, as shown, was used to defibrinate the blood when the flask was hand-swirled for 5 minutes.

Figure 2. A photograph of the cotton-fiber column used for the purification of bovine lymphocytes. The photograph shows (1) the lower plastic tube holder and flow seal; (2) the syringe barrel containing the cotton fiber bed; (3) the syringe plunger for compressing the fiber bed; (4) a sample of the cotton fibers; and (5) the assembled column, prior to compressing the fiber bed for cell elution.
tubing was placed on the needle end of the syringe barrel to aid in subse-
quent removal of cells from the column (Figure 2).

The packed column was washed once with EBSS and then lightly press-
fitted into a plastic centrifuge tube which fit tightly around the base of
the syringe barrel. The column was then covered with aluminum foil and,
together with a separately wrapped plunger, was sterilized by autoclaving
at 121°C for 15 minutes.

Approximately 10 ml of the buffy-coat cell suspension were pipetted
onto the column and washed into the cotton fiber bed with an additional 5
to 10 ml volume of EBSS. The column was then pressed firmly into the plas-
tic tube to form a seal which prevented column flow. Sterile aluminum foil
was replaced on the barrel top to prevent drying of the column.

Following an incubation of 30 to 60 min at 37°C, an additional 10-ml
volume of EBSS was added to each column to aid in eluting the nonadhering
lymphocytes. A sterile syringe plunger was partially inserted into the top
of the column when the syringe barrel was pulled out of the lower plastic
tube. After placing the column over another 50-ml screw-cap centrifuge, a
suspension of purified lymphocytes was obtained by fully compressing the
fiber bed with the plunger.

The erythrocytes were eliminated from this cell suspension by hemolysis,
induced when the suspension was mixed for 2 to 3 min with an equal volume
of Tris-buffered isotonic ammonium chloride (ACT), pH 7.2, prepared accord-
ing to the method of Boyle (1968) as follows: one volume of Tris-buffer
(20.6 g Tris base⁹/liter, adjusted to pH 7.65 with HCl), is mixed with

⁹Tris base: 2-amino-2(hydroxymethyl)-1,3-propanediol; Eastman Kodak
Company, Rochester, New York
nine volumes of 0.83% ammonium chloride, and the final solution was adjusted to pH 7.2 with HCl. ACT gave satisfactory hemolysis without causing any apparent damage to lymphocytes. When the lymphocyte suspension was too heavily contaminated with red cells, however, lysis with ACT produced excessive numbers of red cell "ghosts", or stroma. This led to extensive clumping of the lymphocytes when they were allowed to stand for a period of time prior to their distribution in culture.

The lymphocytes were collected by centrifugation for 10 min at 225 x G, washed once with fresh ACT, washed again in EBSS, and resuspended in growth medium.

**Cell counts**

Cells were counted in a Neubauer Hemocytometer counting chamber. Duplicate counts were made of appropriate sample dilutions, and adjustments were made in the original cell suspensions to provide a final cell concentration which ranged from 3 to 6 x 10^6 cells in a 2.5-ml volume, depending on the requirements of the particular experiment.

**Lymphocyte viability**

The viability of cells to be cultured, or of cells processed during the course of a given experiment, was determined by trypan blue dye exclusion (Pappenheimer, 1917; Hanks and Wallace, 1958) and by maintenance of nuclear integrity in the presence of "Cetrimide" (Stewart and Ingram,

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10 J. T. Baker Chemical Company, Phillipsburg, New Jersey

11 C. A. Hauser and Son, Philadelphia, Pennsylvania

12 Hexadecyltrimethylammonium bromide; Eastman Kodak Company, Rochester New York
One-tenth ml of trypan blue stain, prepared at a concentration of 0.4% in Hank's balanced salt solution, was mixed with 0.5 ml of cell suspension (diluted to approximately 150,000 cells/ml) and was allowed to stand for 10 minutes. A total cell count and a count of unstained cells was made. Assuming those cells which were unstained to be viable, the results were expressed as percent viable cells.

Cetrimide is a mixture of tetradecyl alcohols widely used in industry. Its main application is as an antiseptic and detergent, but it has been used for leukocyte counting with the Coulter electronic counter (Hatch and Balazs, 1961). When used in concentrations of 5 mg/ml of fluid, the cytoplasm of viable human lymphocytes was stripped from the cells, leaving the nuclei morphologically intact (Muñiz et al., 1970). The same technique was used to strip the cytoplasm from nonagglutinated bovine lymphocytes, leaving only viable nuclei intact for counting. The nuclei of phytohemagglutinated lymphocytes, however, were most difficult to separate, even when cetrimide was used at a concentration of 15 mg/ml.

Cell culture techniques

Bovine lymphocyte cell cultures were set up in duplicate and contained from 3 to 6 x 10^6 cells/tube (dependent on the experimental requirements) distributed in a volume of 2.5 ml of Eagle's MEM. Certain experimental cultures were set up in triplicate, as described in the Results. Stimulated lymphocyte cultures received an addition of 0.5 ml of either phytohemagglutinin-M (PHA-M)¹³, to give a final culture concentration of 10 μl of

¹³Bacto-Phytohemagglutinin-M; Difco Laboratories, Detroit, Michigan
reconstituted PHA-M solution/ml of culture; or tuberculin PPD\textsuperscript{14} to a final concentration of 10 \( \mu \text{g} \) of reconstituted PPD solution/ml of culture. Unstimulated control cultures received 0.5 ml additional MEM only, in place of one of the additives just noted, thus bringing all tubes up to a final volume of 3 ml of culture medium. The cultures were incubated at 37\(^\circ\)C at about 30\(^\circ\) from the horizontal in a humidified, 5%-CO\(_2\) atmosphere. Incubation times varied from 6 hr to 6 days, depending on the culture. After 72 hr, 2 ml of the supernatant fluid from each of the cultures remaining were removed by aspiration and replaced with 2 ml of either fresh MEM, or MEM-leu, containing serum. Duplicate tubes from each of the test groups were assayed at each of the several time periods for cellular blastogenic responses, as measured by either cytologic findings or by radioisotope incorporation into newly synthesized DNA, RNA, and protein.

**Isotope incorporation studies**

A convenient way to quantitatively determine the extent of lymphocyte transformation in a given culture is to periodically determine the amount of radiolabel which the cultured cells incorporate into DNA, RNA, and protein following initial stimulation with either PHA or PPD.

Twelve hr prior to the termination of incubation for each time period, three of the four sets of lymphocyte cultures received a pulse of radioactive label, as follows: 1.0 \( \mu \text{Ci} \) of \(^3\)H-T was added to the first set of tubes for analysis of its incorporation into DNA; 1.0 \( \mu \text{Ci} \) of \(^3\)H-U was

\textsuperscript{14} Purified Protein Derivative; Parke, Davis and Company, Detroit, Michigan
added to the second set for incorporation into RNA; and 0.25 μCi of $^{14}$C-leu was added to the third set of tubes for incorporation into newly synthesized protein. A fourth set of cultures received no radioisotopes and was used for determining cell numbers and viability.

Labeled cultures were terminated by centrifuging at approximately 200 x G for 8 min, after which the supernatant fluids were carefully decanted into a radioactive-waste bottle. The cell pellets were washed once with cold phosphate-buffered saline, and then resuspended in the few drops of fluid that remained in the tube when the supernatant was decanted. A small aliquot of this concentrated cell suspension was then smeared on freshly cleaned glass slides and prepared for autoradiographic analysis. The remaining cell pellet was frozen at -20°C for subsequent liquid scintillation preparation.

**Autoradiography of cells**

Glass slides to be used for autoradiography were thoroughly cleaned before use. After being placed on edge in a 30-slot staining rack, the slides were boiled for at least 30 min in a dilute solution of Haemo-Sol Cleanser, rinsed thoroughly with distilled water, and then hot-air dried in an open autoclave.

A small aliquot from each radiolabeled cell culture was placed on each of two freshly cleaned slides, fixed in methyl alcohol for 30 min, and air dried overnight. In absolute darkness, smears were dipped in Kodak NTB-3 nuclear track emulsion¹⁵ (one part nuclear emulsion and one part distilled water at 43°C) and allowed to air dry. When all the slides had been dipped

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¹⁵ Eastman Kodak Company, Rochester, New York
and had dried, they were packed in light-proof, black bakelite slide boxes containing a desiccant. After sealing with black plastic tape, the boxes were wrapped twice in aluminum foil and stored at \(-20^\circ C\) for 5 days to allow for exposure of the emulsion. Exposure was terminated by developing the autoradiograms at \(20^\circ C\) for 2.75 min in Dektol developer\(^{15}\) diluted 1:2 in distilled water. Slides were then fixed for 4 min, rinsed with water for 15 min, and stained through the emulsion with Wright's blood stain (Baserga and Malamud, 1969). Grain counts, made over not less than 200 cells in each preparation, were tabulated into four classes: unlabeled (0 to 3 grains); lightly labeled (4 to 9 grains); moderately labeled (10 to 20 grains); and heavily labeled (>20 grains). The background was found to be much less than one grain per cell area.

**Determination of lymphocyte specific activity**

DNA, RNA, and protein syntheses were measured by the incorporation of \(^3\)H-T, \(^3\)H-U, or \(^{14}\)C-leu, respectively, into acid insoluble material, using a procedure modified from that of Hughes and Caspary (1970).

After freezing and thawing the lymphocyte cell pellets, one drop of normal bovine serum was placed in each tube to act as a precipitating carrier protein in subsequent sample processing. After adding 4 ml of ice-cold 5% trichloroacetic acid (TCA) to each tube, cellular protein was allowed to precipitate for at least 60 min at \(4^\circ C\). As the time factor at this point was not critical, precipitation was extended overnight when necessary. The precipitates were then pelleted by centrifugation, and the supernatants were carefully decanted into radioactive-waste storage bottles. After a second precipitation in an additional 4 ml of cold TCA, most precipitates were dehydrated by a wash in 3 ml of cold absolute methanol.
Those samples in which protein synthesis was to be studied, however, were not resuspended in cold methanol, since some solubility of cellular protein in methanol was expected. All samples were then thoroughly air dried for from 12 to 24 hr. This was found to be necessary as otherwise there was often a small carry-over of moisture into the scintillation fluid which subsequently interfered with the efficiency of sample counting. Dried precipitates were dissolved in 0.5 ml of "Soluene-100" solubilizer\textsuperscript{16} and quantitatively transferred to glass counting vials in two 7-ml washes of specially prepared scintillation fluid. This fluid was prepared as follows: 160 ml of 25X "Permafluor"\textsuperscript{16} were added to 3.8 liters of toluene so that the resulting solution contained, per liter, 5.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP). Following overnight dark adaptation of the sample solutions at 4°C, the beta radioactivities of the samples were counted in a Model 3375 Packard Tri-Carb Scintillation Spectrometer\textsuperscript{16} for at least 20 min in order to achieve a statistically significant gross count. For each sample, both the net counts-per-minute (cpm) and an automatic external standard ratio (AES ratio) were permanently recorded on Spectrometer Data Sheets\textsuperscript{16} by teletype readout. The recorded AES ratio data, subsequently used to correct sample activities for quench and efficiency, was based on automatic comparison of sample activities with a standardized radioactive source incorporated into the scintillation counter itself.

The counting efficiency for each lymphocyte sample vial was determined by comparing the AES ratio of that sample to an efficiency calibration

\textsuperscript{16} Packard Instrument Company, Inc., Downers Grove, Illinois
curve, previously prepared using a standard set of calibration vials for tritium and carbon-14 isotopes. A separate calibration curve for each isotope was prepared each time a different experiment was performed. In order to prepare each isotope's calibration curve, the efficiency of counting of the known activities in the set of standard vials was determined. Counting efficiency was determined by comparing the observed activity (cpm) of each of the standard vials to its known activity in cpm and multiplying by 100. These efficiencies were then plotted against the recorded AES ratio for each standard vial to form a calibration curve for each isotope. The counting efficiency for each of the lymphocyte samples was obtained by fitting the recorded AES ratio for that sample to the prepared curves. Counting efficiencies for most lymphocyte samples were near 30% when labeled with tritium and near 45% when labeled with carbon-14.

Cytologic studies

Lymphocyte cultures were examined for morphologic transformation as evidenced by an increase in the number of large blast cells in cultures stimulated by PHA or PPD. As done by Weber (1966), autoradiographic slide preparations were used for this study. Lymphocytes were arbitrarily classified as small (approximately 6 μ), medium (7 to 12 μ), and large (>12 μ). No less than 200 randomly chosen cells were counted on each slide with determination of the percentage of blast transformation using the morphologic criteria described by Marshall and Roberts (1963b) and by Robbins (1964). All mitotic and blast-like cells were classed as transformed cells.
RESULTS

Several published reports have emphasized that, unless careful attention is paid to all aspects of cell culture and liquid scintillation techniques, enormous variations might occur which would make meaningless the results obtained (Hughes and Caspary, 1970; Phillips and Zweiman, 1970; Fernald and Metzgar, 1971). Factors which could affect the degree of cellular transformation or the uptake of labeled precursors were therefore carefully examined, not only as potential causes of variation, but also to obtain maximum culture responsiveness.

Lymphocyte separation

There are a variety of techniques available which work well in isolating and purifying the lymphocytes from the blood of a large variety of species, including man. These methods proved inadequate, however, for the reproducible separation and purification of bovine lymphocytes. Bovine erythrocytes have a much lower sedimentation rate than those of other species. Separation of bovine erythrocytes and white blood cells by sedimentation at room temperature in any of several density gradients (Ficoll, glucose, gelatin) was not found satisfactory, since the erythrocytes and leukocytes sedimented together. To separate these cell types, then, whole blood was centrifuged in sterile 50-ml tubes at 750 x G for 40 min and theuffy-coat cell-suspension (which formed at the interface between the lower packed red cell volume and the upper serum supernatant) was collected by pipette. By not removing too many red cells with the buffy coat, a fairly clean leukocyte suspension was regularly obtained.
Table 2. Separation of lymphocytes from bovine blood

<table>
<thead>
<tr>
<th>Whole Blood Treatment</th>
<th>Total Number of White Cells per ml of Blood (Average)</th>
<th>Percent Lymphocytes (Average)</th>
<th>Percent Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparinized(^a)</td>
<td>12.6 x 10(^6)</td>
<td>64.5</td>
<td>98.6</td>
</tr>
<tr>
<td>Defibrinated(^b)</td>
<td>7.2 x 10(^6)</td>
<td>80.7</td>
<td>96.4</td>
</tr>
<tr>
<td>Final Column Separation</td>
<td>13.6 x 10(^7)</td>
<td>98.6</td>
<td>94.9</td>
</tr>
</tbody>
</table>

\(^a\) Ten U. S. P. units of heparin (ammonium salt) added/100 ml blood

\(^b\) See Text

Clotting of blood taken from test animals was prevented by manually defibrinating the blood as described earlier, rather than by using an anticoagulant. Adequate defibrination of the blood was essential to prevent formation of clots and subsequent loss of cells. Complete defibrination could not be achieved if the blood-collecting bottle was too full, if hand-swinging of the flask was too slow, or if the defibrinating sticks were loose in the flask. Defibrination of the blood served to remove most, if not all, of the blood platelets, and a good percentage of the granular leukocytes, as is shown in Table 2. This table shows the averaged results from 5 animals. Although the total leukocyte yield was lower in defibrinated blood, the lymphocyte concentration was almost 20% higher than in heparinized blood. Further, the 5 to 10 min defibrination procedure was not injurious to the cells. As shown in Table 2, the average viability as determined by trypan blue dye exclusion was very high (about 96%) and was only slightly lower than the viability of the more gently-treated hepari-
Lymphocytes and granular leukocytes were separated by filtering the buffy-coat cell suspension through a cotton-fiber column. Cotton fibers, pulled from one-inch squares of cheesecloth, were found to be superior to other materials for reproducible separation of lymphocytes. Columns prepared with glass wool, glass beads, or even nylon fibers, were tried, but none worked as well or as reproducibly as did the cheesecloth fibers.

Following column purification, a small volume of from 15 to 25 ml of purified cell suspension was obtained which contained about $1 \times 10^8$ leukocytes/ml, of which 98 to 99% were lymphocytes (Table 2, and Figures 3 and 4).

Factors affecting lymphocyte transformation

To determine the optimal conditions for obtaining the maximum uptake of radiolabel into newly synthesized DNA, RNA, and protein, a number of conditions which were known to influence cell growth and viability were examined.

Initial viability of cells

Trypan blue dye-exclusion tests showed that about 95% of the total cell population were viable immediately after column separation (Table 2).

Viability at pulse labeling period

The degree of transformation and incorporation of radiolabel into lymphocyte cultures at a given labeling period depended on the proportion of surviving lymphocytes at that time. Using both dye exclusion tests and cetrimide counts of viable nuclei, viability of all cultures was determined at the time of harvest on each of 6 consecutive days for PHA, PPD, and
Figure 3. A photomicrograph of purified bovine peripheral blood lymphocytes after final column separation. X 833

Figure 4. A higher magnification photomicrograph of several lymphocytes from a suspension of purified bovine peripheral blood lymphocytes. X 1170
control cultures. The lymphocytes in PHA-treated cultures tended to be heavily clumped, making accurate viability determinations of individual cells extremely difficult. Even the use of cetrimide, a chemical detergent, did not result in improved viability estimations, as reflected by the low values recorded in Table 3. Table 3 shows a compilation of percent viable cells for eight cell cultures, each measured at the time of harvest. Results indicate that, although cell viability was as high as 95% on day 1, the number of viable cells decreased to approximately 28% on day 6.

The effect of varied PHA and PPD concentrations

The blastogenic response of bovine lymphocytes in vitro was found to be dependent on the concentration of phytohemagglutinin or tuberculin antigen used to activate the culture. A variety of stimulant levels were used to determine the appropriate concentration which would provide maximal blastogenic stimulation of lymphocytes as measured by cellular incorporation of $^3$H-T into newly synthesized DNA.

**Phytohemagglutinin.** Lymphocytes from seven animals were tested for response to doses of PHA which ranged from 0 to 100 μl PHA/ml of culture medium (Figure 5). The lymphocytes were cultured at a concentration of $10^6$ cells/ml for 72 hr, after which $^3$H-T was added for 12 hr.

Bovine lymphocytes showed a definite dose-dependent response to PHA stimulation. Maximum $^3$H-T uptake was stimulated by PHA concentrations which ranged from 5 to 15 μl PHA/ml of culture. Concentrations of PHA greater than 15 μl PHA/ml in the culture media resulted in a decreased isotope incorporation. A concentration of 10 μl PHA/ml of culture medium was therefore selected for subsequent experiments.

There was no observed difference in the pattern of response to PHA
Table 3. Lymphocyte viability as a percent of the total number of cells at the time of culture termination (mean value and range)

<table>
<thead>
<tr>
<th>Day of Harvest (days)</th>
<th>Type of culture treatment and method of viability determination</th>
<th>Total Combined Average Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA (cetrimide)</td>
<td>PPD (cetrimide)</td>
</tr>
<tr>
<td>.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>37 (25-64)</td>
<td>60 (40-85)</td>
</tr>
<tr>
<td>2</td>
<td>31 (21-50)</td>
<td>48 (30-99)</td>
</tr>
<tr>
<td>3</td>
<td>21 (14-31)</td>
<td>31 (13-49)</td>
</tr>
<tr>
<td>4</td>
<td>17 (10-26)</td>
<td>30 (11-51)</td>
</tr>
<tr>
<td>5</td>
<td>17 (10-23)</td>
<td>25 (10-51)</td>
</tr>
<tr>
<td>6</td>
<td>16 (10-25)</td>
<td>25 (10-43)</td>
</tr>
</tbody>
</table>
Figure 5. A graph of the dependency of bovine lymphocyte stimulation on the dose of phytohemagglutinin. Lymphocyte suspensions were incubated with PHA-M for 72 hr; $^{3}$H-thymidine incorporation was determined after an additional incubation of 12 hr with the isotope. Each point represents the mean of triplicate determinations. Lymphocytes from both tuberculin-sensitive and nonsensitive (control) animals were examined, and were:

**Tuberculin-sensitive animals**

<table>
<thead>
<tr>
<th>#</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>△</td>
</tr>
<tr>
<td>56</td>
<td>♦</td>
</tr>
<tr>
<td>58</td>
<td>■</td>
</tr>
<tr>
<td>63</td>
<td>△</td>
</tr>
<tr>
<td>67</td>
<td>□</td>
</tr>
</tbody>
</table>

**Tuberculin-nonsensitive animals (controls)**

<table>
<thead>
<tr>
<th>#</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1600</td>
<td>●</td>
</tr>
<tr>
<td>1626</td>
<td>○</td>
</tr>
</tbody>
</table>
between lymphocytes obtained from tuberculin-sensitized or nonsensitized (control) animals.

**Tuberculin.** Six tuberculin-sensitive animals and one nonsensitive animal were tested for response to varying doses of PPD antigen ranging in concentration from 2 to 200 µg PPD/ml of culture medium (Figure 6). Cultures were set up at a concentration of $1 \times 10^6$ cells/ml and were terminated on day 4 after a 12-hr incubation with $^3$H-T. Although not as strong as the PHA response, the response of tuberculin-sensitive lymphocytes to increased concentrations of PPD was dose-dependent. Maximum stimulation of isotope uptake occurred at a concentration of from 5 to 20 µg PPD/ml of culture medium. Use of concentrations higher than 20 µg PPD/ml resulted in decreased total isotope incorporation. A concentration of 10 µg PPD/ml of culture medium was selected for subsequent experiments.

Limited isotope incorporation occurred in the control cultures at any level of the antigen. At high concentrations of PPD, isotope incorporation into control cultures was suppressed when compared to uptake at lower levels of PPD. A wide range of responses was noted in the tuberculin-activated cultures. For example, cells from animal no. 27 showed a high isotope incorporation at low PPD concentrations while lymphocytes from animal no. 54 showed only a low isotope incorporation even at high levels of PPD. This reflected the individual variation in tuberculin sensitivity of the different animals.

**Cell concentration**

Variations in the density of cells contained in PHA- and PPD-treated cultures influenced the amount of $^3$H-T which these cultures incorporated. Two experiments were conducted which determined the extent of this variation.
Figure 6. A graph of the dependency of bovine lymphocyte stimulation on the dose of antigen. Lymphocyte suspensions from sensitized and nonsensitized (control) animals were incubated with tuberculin PPD for 96 hr; \(^3\text{H}\)-thymidine incorporation was determined after an additional incubation of 12 hr with the isotope. Each point represents the mean of triplicate determinations. Lymphocytes from both tuberculin-sensitive and nonsensitive (control) animals were examined. Lymphocyte donors were:

**Tuberculin-sensitive animals**

- #19
- #27
- #54
- #56
- #67
- #8854

**Tuberculin-nonsensitive animal (control)**

- #1626
PPD dose response

Counting rate, counts per minute

(µg PPD/ml of culture)
Cells from two animals were cultured at four different concentrations: 0.5, 1, 2, and $3 \times 10^6$ cells/ml of culture (Figure 7).

PHA-treated cultures incorporated isotope in a direct linear response to increasing cell densities from 1 to $3 \times 10^6$/ml of culture medium. This linear relationship indicated that there was not an optimal cell density which resulted in the most efficient uptake of radioisotope, since $^3$H-T incorporation increased in direct proportion to the increasing number of lymphocytes.

The PPD-stimulated cultures incorporated from 5 to 30 times less isotope than did those cultures containing PHA. The PPD cultures, however, showed a definite optimum at approximately $2 \times 10^6$ cells/ml, at which cell density the cultures showed a higher uptake of label than was observed in those cultures containing 1 or $3 \times 10^6$ cells/ml of culture medium.

On the basis of these results, all cultures in subsequent experiments were set up using a cell density of from 1 to $2 \times 10^6$ cells/ml where possible.

**Nature and concentration of serum used in cultures**

Lymphocytes from 4 animals were used to study the effects which varying concentrations of autologous serum or newborn calf serum had on isotope incorporation in PHA-stimulated cultures (Figures 8 and 9). Lymphocyte suspensions were prepared in the usual way and cultures were set up in triplicate. Newborn calf serum (NBCS) was tested at a final culture concentration of 5, 10, 20, and 40% (v/v). Autologous serum was tested at a final culture concentration of 20 and 40% (v/v). A 10% supplement of dextran to 10% of NBCS and, in other tubes, to 10% autologous serum was also investigated for its suitability as a serum substitute. Cultures were terminated on the third day.
Figure 7. A graph of the effect which varied cell concentrations had on the measured uptake of $^3$H-thymidine by stimulated bovine lymphocytes. Cells were incubated with 10 μl PHA/ml of medium for 84 hr. Label was added at 72 hr. Each point represents the mean of triplicate determinations. The lymphocyte donor and the culture were:

Animal no. 19

- PHA - PPD

Animal no. 67

- PHA - PPD
Effect of cell concentration on isotope uptake.
Figure 8. A graph of the effect which varied concentrations of newborn calf serum had on the uptake of $^{3}$H-thymidine into PHA-stimulated cells. The individual responses, and an averaged response, of 4 animals are shown:

#67
#21
#27
#19
Average

Figure 9. A bar graph comparing the effect of varied concentrations of newborn calf serum or autologous serum on the uptake of $^{3}$H-thymidine into PHA-stimulated cultures. Each bar represents the averaged response of 4 animals.

NB - newborn calf serum (NBCS)
NBX - 10% NBCS + 10% dextran
A - autologous serum (AS)
AX - 10% AS + 10% dextran
(Corrected counting rate, cpm)
The $^3$H-T uptake was found to increase as additional newborn calf serum was added up to 20%, after which the isotope uptake dropped off slightly in 3 of 4 cultures. Less than one-third the isotope uptake at 10% serum concentration was observed at 5% serum concentration.

In all cases, the use of autologous serum resulted in a lower isotope uptake than was observed in comparative cultures using newborn calf serum. Cultures containing 10% NBCS showed higher $^3$H-T incorporation, for example, than did cultures containing 20% autologous serum, and cultures with 20% NBCS had higher labeling than did cultures containing 40% autologous serum.

The addition of 10% dextran to the culture medium was found to slightly decrease the isotope uptake of a given culture as compared to cultures without its addition. As a serum substitute, dextran was not found to be suitable in enhancing growth of stimulated cultures.

Culture requirements for L-glutamine, nonessential amino acids, or sodium pyruvate

Much of the commercial media available today is prepared without added L-glutamine. Several experiments were therefore designed to investigate the effect which the addition of L-glutamine had on isotope incorporation into PHA-stimulated cultures.

Lymphocyte cultures from three animals were set up in triplicate and were examined for in vitro proliferative response when grown either in medium without L-glutamine; with only L-glutamine added; or with L-glutamine, non-essential amino acids, and sodium pyruvate added. In addition, two different serum concentrations were examined in the cultures containing added L-glutamine. The results are tabulated in Table 4.

The addition of L-glutamine to a final culture concentration of 2 mM/ml
Table 4. The effect which culture medium additives had on the uptake of \(^{3}H\)-thymidine by PHA-stimulated cells

<table>
<thead>
<tr>
<th>Lymphocyte donor</th>
<th>Culture medium</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
<td>M2</td>
<td>M3</td>
<td>M4</td>
</tr>
<tr>
<td>51</td>
<td>20,518</td>
<td>43,256</td>
<td>40,301</td>
<td>22,277</td>
</tr>
<tr>
<td>53</td>
<td>28,433</td>
<td>58,463</td>
<td>49,296</td>
<td>25,564</td>
</tr>
<tr>
<td>54</td>
<td>35,037</td>
<td>72,557</td>
<td>61,447</td>
<td>41,320</td>
</tr>
<tr>
<td>Donor average</td>
<td>27,996</td>
<td>58,092</td>
<td>50,348</td>
<td>29,720</td>
</tr>
</tbody>
</table>

M1 = Minimum Essential Medium (MEM) + 20% NBCS  
M2 = MEM + 20% NBCS + L-glutamine (2 mM/ml)  
M3 = MEM + 20% NBCS + L-glutamine (2 mM/ml) + nonessential amino acids (1/100 dilution) + sodium pyruvate (1/100 dilution)  
M4 = MEM + 30% NBCS + L-glutamine (2 mM/ml)

more than doubled the isotope uptake into PHA-stimulated cultures compared to cultures which did not receive added L-glutamine but which contained the same serum concentration (20% NBCS, v/v). When serum concentration in cultures containing L-glutamine was raised to 30% NBCS (v/v), however, a large decrease in the incorporation of isotope was noted, which closely paralleled the response of those cultures with no added L-glutamine. This data supported the studies on serum concentrations just presented which indicated that 20% NBCS (v/v) resulted in optimal isotope incorporation in stimulated cultures.

Addition of nonessential amino acids and sodium pyruvate to cultures already containing L-glutamine did not result in improved isotope incorporation. On the contrary, these additives were found to decrease the overall culture responses rather than enhance them.
In control cultures, spontaneous isotope incorporation was evaluated as it was affected by the source and concentration of serum, and the inclusion of additives such as L-glutamine into the culture medium. Control cultures in media containing NBCS showed slightly higher rates of spontaneous isotope incorporation than those cultures which contained autologous serum, although the difference was small. Further, those control cultures which contained added nonessential amino acids, and sodium pyruvate showed an increased rate of spontaneous isotope uptake when compared to similar cultures without these additives.

As a result of these studies, bovine lymphocyte cultures were routinely prepared to contain 20% NBCS (v/v) and added L-glutamine (2 mM/ml).

The presence or absence of leucine in the culture medium

Protein synthesis by stimulated lymphocytes was determined by measuring the amount of $^{14}$C-leucine incorporated into cells cultured in Minimum Essential Medium without leucine (MEM-leu). Two experiments were performed to determine if the use of MEM-leu would result in a decreased cellular responsiveness, as would be shown by a decreased incorporation of $^{3}$H-T label into newly synthesized DNA.

Lymphocyte cultures were prepared in duplicate in two different media: either MEM with leucine or MEM without leucine. After 72 hr incubation with PHA, $^{3}$H-T was added for an additional 12 hr. The cultures were then terminated and isotope incorporation was analyzed by liquid scintillation. The incorporation of label into newly synthesized DNA is presented in Table 5.

The results indicate that no detrimental effect on cellular responsiveness occurred when leucine was removed from the culture medium.
Table 5. The effect which the presence or absence of leucine had on the blastogenic response of lymphocytes to PHA stimulation in vitro

<table>
<thead>
<tr>
<th>Culture Treatment</th>
<th>Animal</th>
<th>27</th>
<th>854</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MEM + leu</td>
<td>MEM - leu</td>
</tr>
<tr>
<td>PHA</td>
<td></td>
<td>223,921</td>
<td>255,491</td>
</tr>
<tr>
<td>PPD</td>
<td></td>
<td>25,112</td>
<td>47,995</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td>947</td>
<td>968</td>
</tr>
</tbody>
</table>

The time course of PHA- and PPD-stimulated cultures

Once optimal culture conditions had been determined, several experiments were designed to determine that time period after PHA or PPD stimulation when the maximum lymphocyte response occurred, as indicated by uptake of $^3$H-T into newly synthesized DNA.

Phytohemagglutinin

In two separate experiments, lymphocyte cultures from eight animals were terminated and processed for isotope evaluation beginning one day after initial stimulation and thereafter at 24-hr intervals. Figure 10 illustrates the time course of the lymphocyte proliferative response from four animals in the first trial.

The peak response of these lymphocytes occurred on day 2 (48 hr post-stimulation); the observed response on day 3 also showed good isotope incorporation. A rapid drop in culture activity was observed between days 3 and 4, however. On day 4 the medium on all cultures was restored with fresh
Figure 10. A graph of the amount of isotope incorporated by bovine lymphocytes as determined on each of 7 consecutive days following initial PHA stimulation. Each point represents the mean of triplicate determinations. Cells were cultured at a density of 10^6 cells/ml in Eagle's MEM which contained 10 µl PHA-M/ml. Tritiated-thymidine was present for the final 12 hr of culture incubation. Fresh medium was added to all remaining cultures on day 4. Lymphocytes were taken from the following animals:

#64
#55
#66
#61
The time course of PHA stimulation

Counting rate, counts per minute

(Days following stimulation)

Change of medium
medium, as follows: 2 of the 3 ml of culture medium in each tube were aseptically removed by aspiration and immediately replaced with 2 ml of fresh medium containing no mitogen.

The change in medium did little to restore or enhance lymphocyte activity. A slight plateau in isotope incorporation was observed between days 4 and 5, but then the proliferative response dropped rapidly until all remaining cultures were terminated.

The second experiment (Figure 11), also set up with lymphocytes from four animals, was similar to the first trial except that the culture medium was changed on day 3 rather than on day 4. This earlier change of medium resulted in a large difference in the ability of stimulated cultures to incorporate specific label on days 4 through 6. Although an early peak of isotope uptake was observed on day 2, a second peak of isotope incorporation was observed beginning immediately following the change in culture medium. This second peak not only showed a more sustained isotope uptake, but it also exhibited a doubling in lymphocyte uptake of $^3$H-T. This response, in the absence of any added mitogen, was observed in all subsequent PHA-stimulated cultures whose medium was changed on day 3.

No experiments were performed in which medium was changed on day 2 (the first activity peak), and again on day 4 (the second peak), to determine if a continued increase in isotope incorporation could be achieved.

**Tuberculin**

Lymphocytes from five sensitized and two nonsensitized animals were tested for their in vitro response to PPD stimulation during 6 days of culture (Figure 12). Triplicate cultures were terminated at 24-hr intervals.
Figure 11. A graph of the amount of isotope incorporated by bovine lymphocytes as determined on each of 6 consecutive days following initial PHA stimulation. Each point represents the mean of triplicate determinations. Cells were cultured at a density of $10^6$ cells/ml in Eagle's MEM which contained 10 µl PHA-M/ml. Tritiated-thymidine was present for the final 12 hr of culture incubation. Fresh medium was added to all remaining cultures on day 3. Lymphocytes were taken from the following animals:

#19  ●
#21  ○
#25  ▲
#48  △
Figure 12. A graph of the amount of isotope incorporated by sensitized and nonsensitized bovine lymphocytes as determined on each of 6 consecutive days following initial contact with antigen. Cells were cultured at a density of $10^6$ cells/ml in Eagle's MEM which contained 10 μg PPD/ml. Tritiated-thymidine was present for the final 12 hr of incubation of each culture. Fresh medium was added to all remaining cultures on day 3. Each point represents the mean of triplicate determinations. Lymphocytes were taken from the following animals:

**Tuberculin-sensitive animals**

- #19
- #21
- #25
- #29
- #67

**Tuberculin-nonsensitive animals (controls)**

- #1626
- #1654
The time course of PPD stimulation

(Counting rate, counts per minute) →

(Days following stimulation) →
Maximum isotope incorporation by lymphocytes from sensitized animals occurred on the 5th and 6th days of culture, after which time all cultures were terminated.

Lymphocyte cultures from nonsensitized donors did not respond to PPD antigen in vitro with an increased isotope incorporation at any time. As early as day 2, sensitized cultures showed greater isotope uptake than the nonsensitized cultures.

The wide range of responses in PPD-stimulated cultures again reflects the heterogeneity and individuality of donor sensitivity to a particular antigen, which in this case was tuberculin PPD.

The slow increase in isotope uptake after PPD stimulation is in sharp contrast to the rapid and early rise in lymphocyte activity following PHA stimulation. Consequently, the change of culture medium on day 3 had much less of an observable effect on PPD-stimulated cells than it did on PHA-stimulated cells.

Formation of uropods by bovine lymphocytes in vitro

Numerous "tail-like" uropods, of a wide variety of sizes and shapes, were observed on bovine lymphocytes cultured in vitro (Plate 1, A to F), giving them a "hand-mirror", or polarized configuration which has often been described in the literature (McFarland et al., 1966; Biberfeld, 1971). At the most, 5 to 12% of the lymphocytes examined in a given culture showed this structure (from 54 to 119 uropods/1,000 cells), with almost as many being found in control as in stimulated cultures (Figure 13).

Uropods varied considerably with respect to length and thickness. The
Plate 1. Photomicrographs of uropods observed in cultures of stimulated and unstimulated bovine lymphocyte cultures.

A to E  Variations in the structure of uropods in unstimulated lymphocytes

F     Large uropod of a PHA-stimulated blast

X 1170
thickness of the uropod was in general inversely proportional to its length and often tapered toward its distal end (Plate 1, C). Uropods on blast cells in stimulated cultures, however, tended to be thicker and "heavier" appearing (Plate 1, F) than the small thin "tails" seen on unstimulated cells (Plate 1, A to C). The latter often had a rounded, bulbous end which might have indicated an attachment to the glass slide or culture tube.

The uropod in all cases stained a light blue with Wright's stain, and revealed little internal structure. The posterior nuclear end of the cell stained an intense, dark blue-black. The nucleus was most often rounded and slightly irregular or elongated. Occasionally, a few smaller pseudopods
were observed near the nucleus on the side opposite the uropod.

In contrast to the report of Biberfeld (1971), fewer uropods were noted in PHA-stimulated cultures than were seen in PPD-treated cultures or even control cultures.

No cell-to-cell contacts via the uropod were observed in the specimens examined.

The relationship between DNA, RNA, and protein synthesis and morphologic blast transformation

Following initial in vitro stimulation with PHA or PPD, lymphocyte cultures from seven animals were examined and compared for simultaneous cellular uptake of $^3$H-T, $^3$H-U, or $^{14}$C-leu into newly synthesized DNA, RNA, or protein molecules, respectively. Cultures were terminated at 6, 12, and 24 hr after initial stimulation, and every 24 hr thereafter through the 6th day. Isotope uptake was measured by liquid scintillation analysis of radiolabel incorporated into acid-insoluble cellular precipitates (Figures 14 to 20) and by autoradiography of smears made daily from cultured cells (Figures 21 to 25).

Liquid scintillation determinations

The in vitro response of lymphocytes from seven animals to stimulation by PHA and PPD was determined by liquid scintillation analysis of isotope incorporation into acid-insoluble cellular precipitates. The results are presented in Figures 14 to 20. Each recorded counting rate was adjusted to a rate equivalent to a culture containing $10^6$ viable cells/ml of culture; and corrected for efficiency and quench in the scintillation spectrometer,
Figure 14. A graph of the *in vitro* relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 19. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

**PHA-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[ ]</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

**PPD-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[ ]</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

- [ ] [ ] [ ]
Response of animal no. 19

(Days following stimulation)

(Corrected counting rate, cpm, per 10^6 cells)
Figure 15. A graph of the *in vitro* relationship between ³H-thymidine, ³H-uridine, and ¹⁴C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 21. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

**PHA-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>○</td>
<td>△</td>
<td>□</td>
</tr>
</tbody>
</table>

**PPD-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPD</td>
<td>•</td>
<td>△</td>
<td>■</td>
</tr>
</tbody>
</table>
- (Corrected counting rate, cpm, per $10^6$ cells) -

(Days following stimulation)

Response of animal no. 21
Figure 16. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 25. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

<table>
<thead>
<tr>
<th>Phenol fostered cultures</th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA-stimulated cultures</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
</tr>
<tr>
<td>PPD-stimulated cultures</td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
<td><a href="#">Diagram</a></td>
</tr>
</tbody>
</table>
Figure 17. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 27. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

**PHA-stimulated cultures**

- DNA
- RNA
- Protein

**PPD-stimulated cultures**

- DNA
- RNA
- Protein
Figure 18. A graph of the *in vitro* relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 48. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

<table>
<thead>
<tr>
<th>PHA-stimulated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
</tr>
<tr>
<td>RNA</td>
</tr>
<tr>
<td>protein</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPD-stimulated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
</tr>
<tr>
<td>RNA</td>
</tr>
<tr>
<td>protein</td>
</tr>
</tbody>
</table>
Corrected counting rate, cpm, per 10^6 cells

Days following stimulation

Response of animal no. 48

100
Figure 19. A graph of the *in vitro* relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 67. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

**PHA-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><a href="#">---</a></td>
<td><a href="#">△</a></td>
<td><a href="#">□</a></td>
</tr>
</tbody>
</table>

**PPD-stimulated cultures**

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>protein</th>
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Figure 20. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures from animal no. 854. Cellular responses on each of 6 consecutive days were determined by liquid scintillation analysis of the isotopes incorporated into acid-insoluble cellular precipitates. Isotopes were added to separate cultures and incubated for 12 hr. Each point represents the average of duplicate determinations. The culture responses are recorded as follows:

**PHA-stimulated cultures**

- DNA
- RNA
- Protein

**PPD-stimulated cultures**

- DNA
- RNA
- Protein
Response of animal no. 854

Corrected counting rate, cpm, per 10^6 cells

(Days following stimulation)
corrected for background counting rate and, lastly, corrected by subtracting control from stimulated culture values. This manipulation of the data served to standardize and equalize the results so that their comparison would be more meaningful.

**PHA-stimulated cultures.** Lymphocytes from all animals showed a strong and rapid uptake of specific radiolabel when stimulated in vitro with PHA. As early as 6 hr after stimulation, significant levels of RNA and protein labeling were detectable. In 5 of 7 experiments (cultures 25, 27, 48, 67, and 854), RNA synthesis preceded protein and DNA synthesis, as indicated by a greater uptake of label within the first 24 hr after cellular activation. In all cultures, initial labeling of RNA exceeded that of protein. In 6 of 7 cultures, RNA labeling was more than twice that of protein labeling.

The peak of $^3$H-U uptake varied from culture to culture. Maximum early activity was found at 24 hr (cultures 48 and 67) and 48 hr (cultures 19, 21, and 854). Later peaks occurred on days 4 or 5 (cultures 19, 21, 25, 27, and 48), or day 6 (culture 67). A similar heterogeneous response was noted in the uptake of $^{14}$C-leu into protein, which quite closely paralleled the RNA response at most of the time intervals, although at a lower activity level.

In 5 of 7 experiments (cultures 25, 27, 48, 67, and 854) there was no significant DNA labeling until day 2. By that time, however, from 5 to 30 times as much label was incorporated into DNA as was taken up into either RNA or protein. Incorporation of $^3$H-T into DNA in PHA-stimulated cultures showed two peaks of activity: early uptake reached a maximum at 2 days and dropped off just prior to the change of medium on day 3. After the culture medium was changed, a second peak in thymidine uptake occurred on days 4 to 5. The activity in this peak was more than twice that detected on day 3.
This pattern of response was supported by grain counts of autoradiographs prepared from cultures which had been incubated with $^3$H-T. Lymphocytes from animals 27 and 854 did not show this second peak response. These latter two animals showed only one peak of DNA labeling in spite of the medium change, and this maximum occurred on day 3 for cells from animal 854 and day 4 for cells from animal 27.

The change to fresh medium on day 3 influenced the RNA and protein uptake of label as it did the uptake of isotope into DNA. An increase in $^3$H-U and $^{14}$C-leu incorporation occurred in most cultures following this change in medium (cultures 19, 21, 48, and 67), reversing a detected downward trend in activity from day 2 to day 3.

The rate of uptake of $^{14}$C-leu into protein stabilized after day 2 in several cultures (culture 25, 48, and 67). The rate of incorporation of $^3$H-U became fairly stable after 48 hr in culture 21.

Isotope uptake into both RNA and DNA, as measured by both liquid scintillation and autoradiography, decreased sharply in most PHA-stimulated cultures after day 4 or 5. Incorporation of radiolabel into protein, however, continued to rise throughout the culture period in cultures 19, 27, and 67 and was at a maximum on day 6 when the cultures were terminated. In cultures 27 and 854, $^{14}$C-leu incorporation rose sharply from day 5 to day 6. A similar increase in DNA and RNA labeling in this same time period was observed in culture 27.

**PPD-stimulated cultures.** The *in vitro* response of bovine lymphocytes to PPD stimulation occurred later than the PHA-stimulated response and exhibited a decreased isotope incorporation. Unlike the lymphocyte response to PHA, however, isotope incorporation in PPD cultures continued to increase
after days 1 and 2 and, in most experiments, reached a maximum in RNA, DNA and protein at the termination of the cultures on day 6. Only lymphocytes from animal 854 exhibited a large decrease in PPD responsiveness after day 4; a significant decrease in PHA responsiveness in cultures from this same animal was detected 24 hr earlier.

RNA and protein label incorporation preceded DNA label uptake in tuberculin-stimulated lymphocytes. As early as 24 hr after initial contact with antigen, incorporation of $^3$H-U exceeded that of $^3$H-T or $^{14}$C-leu in all cultures except 854. The amount of $^{14}$C-leu incorporated into protein was slightly less than the amount of isotope taken up into RNA, but was greater than the amount taken up into DNA. By 48 to 72 hr, however, DNA incorporation of $^3$H-T had surpassed incorporation of either $^3$H-U or $^{14}$C-leu and continued to exhibit greatest activity at the termination of the cultures.

By days 5 and 6, isotope incorporation in several PPD-stimulated cultures was nearly identical to that detected in PHA-stimulated cultures (cultures 19, 21, 48, and 67). On days 5 and 6, PPD-activated DNA labeling was from 3 to 30 times greater than protein labeling and from 1.5 to 6 times greater than RNA labeling.

Except in cultures 19, 27, and 854, the overall rate of $^3$H-T, $^3$H-U, and $^{14}$C-leu incorporation by PPD-stimulated cell cultures continued to rise throughout the entire culture period. This response to tuberculin antigen was confirmed by grain counts in autoradiographs prepared from the same stimulated cultures. Activity of all three isotopes dropped between days 5 and 6 in cultures from animal 19, while in cultures 27 and 854, a more pronounced leveling or decrease in isotope incorporation occurred after day 3.
Autoradiography

Culture samples from five animals were prepared for analysis by autoradiography as previously described. No less than 200 cells were examined in each autoradiograph and classified according to the number of grains detectable over each cell. All lymphocytes labeled with 10 or more grains were classified as being activated or transformed cells.

Autoradiographs of radiolabeled blast-transformed cells are presented in Plate 2. Photograph F in Plate 2 illustrates the range of cellular labeling which was observed in many autoradiographic preparations. The grain counts over the four cells pictured indicated an isotope labeling which ranged from light to medium to heavy.

PHA-stimulated cultures were very difficult to quantitate due to the extensive lymphocyte clumping which regularly occurred in the presence of phytohemagglutinin (Plate 2, A and B). As these photographs show, cellular labeling in a given cluster ranged from heavy to light, with many cells possessing no grains at all. In many cases cellular labeling was so intense that the labeled cell was totally obscured (Plate 2, C to E).

The results of grain counts are presented in Figures 21 to 25. In each of these figures, three sets of three lines each are recorded on linear plots, and represent DNA, RNA, and protein synthesis in PHA, PPD, and control cultures. The synthetic activity of each culture was recorded as the percent of cells which had greater than 10 grains.

**PHA-stimulated cultures.** A greater proportion of cells were heavily labeled when cultured with $^{14}$C-leu than were labeled when cultured with either $^3$H-T or $^3$H-U. On day 1, from 35 to 62% of the cells which incorporated $^{14}$C-leu had more than 10 grains. At the same time, only 18 to 48%...
Plate 2. Autoradiographs of PHA- and PPD-stimulated bovine blood lymphocytes.

A to B  PHA-stimulated, agglutinated lymphocytes; with cellular labeling ranging from light to heavy, and including many unlabeled cells; \(^3\text{H}\)-thymidine label.  
X 260

C to E  PHA-stimulated lymphocytes, illustrating the size of large blast-cells compared to unresponsive small lymphocytes; \(^3\text{H}\)-thymidine label.  
X 1170

F  PPD-stimulated cells, showing light to heavy labeling of lymphocytes; \(^3\text{H}\)-uridine label.  
X 1170
Figure 21. A graph of the *in vitro* relationship between $^{3}$H-thymidine, $^{3}$H-uridine, and $^{14}$C-leucine incorporation into DNA, RNA, and protein, respectively, by PHA- and PPD-stimulated cultures from animal no. 19. Cellular responses on each of 6 consecutive days were determined by grain counts which were made over not less than 200 randomly chosen cells in each autoradiographic preparation. Results were adjusted to express the percentage of cells, per 1,000, with a grain count greater than 10. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
Autoradiography - animal no. 19

DNA synthesis

RNA synthesis

protein synthesis

(Days following stimulation)
Figure 22. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation into DNA, RNA, and protein, respectively, by PHA- and PPD-stimulated cultures from animal no. 21. Cellular responses on each of 6 consecutive days were determined by grain counts which were made over not less than 200 randomly chosen cells in each autoradiographic preparation. Results were adjusted to express the percentage of cells, per 1,000, with a grain count greater than 10. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
Autoradiography - animal no. 21

- DNA synthesis
- RNA synthesis
- Protein synthesis

(Number of cells per 1000, with >10 grains)

(Days following stimulation)
Figure 23. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation into DNA, RNA, and protein, respectively, by PHA- and PPD-stimulated cultures from animal no. 25. Cellular responses on each of 6 consecutive days were determined by grain counts which were made over not less than 200 randomly chosen cells in each autoradiographic preparation. Results were adjusted to express the percentage of cells, per 1,000, with a grain count greater than 10. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
Figure 24. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation into DNA, RNA, and protein, respectively, by PHA- and PPD-stimulated cultures from animal no. 48. Cellular responses on each of 6 consecutive days were determined by grain counts which were made over not less than 200 randomly chosen cells in each autoradiographic preparation. Results were adjusted to express the percentage of cells, per 1,000, with a grain count greater than 10. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
Figure 25. A graph of the in vitro relationship between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation into DNA, RNA, and protein, respectively, by PHA- and PPD-stimulated cultures from animal no. 67. Cellular responses on each of 6 consecutive days were determined by grain counts which were made over not less than 200 randomly chosen cells in each autoradiographic preparation. Results were adjusted to express the percentage of cells, per 1,000, with a grain count greater than 10. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
Autoradiography - animal no. 67

DNA synthesis
RNA synthesis
protein synthesis

(Number of cells, per 1000, with >10 grains)
(%) of cells containing >10 grains

(Days following stimulation)
of the cells showed a similar labeling with \( ^3\text{H-U} \), while only from 1 to 5\% of the cells were heavily labeled with \( ^3\text{H-T} \).

Maximum grain counts occurred in the period from 2 to 5 days after initial PHA stimulation. By the 4th to 6th day of culture, from 60 to 97\% of the cells stimulated were heavily labeled with \( ^{14}\text{C-leu} \). At the same time, a maximum of from 27 to 75\% of the cells exhibited heavy labeling with \( ^3\text{H-U} \), while only from 25 to 42\% were heavily labeled by \( ^3\text{H-T} \). In most cultures, the number of grains from all three isotopes declined on the last day of incubation. The medium change on day 3 improved the cellular labeling by one or more of the isotopes in each experiment.

In contrast to results obtained by liquid scintillation, data from autoradiographs indicated that a much greater overall amount of \( ^{14}\text{C-leu} \) was incorporated into cells than of \( ^3\text{H-T} \) or \( ^3\text{H-U} \). Compared to \( ^{14}\text{C-leu} \), \( ^3\text{H-U} \) incorporation was intermediate, while \( ^3\text{H-T} \) exhibited the least incorporation in autoradiographic preparations.

**PPD-stimulated cultures**

Fewer cells were found to be heavily labeled in autoradiographs prepared from cultures of PPD-stimulated cells than were observed in similar preparations from PHA-stimulated cultures. Like the latter, however, PPD-treated cells which had been incubated with \( ^{14}\text{C-leu} \) showed greater labeling than cells incubated with either \( ^3\text{H-T} \) or \( ^3\text{H-U} \). From 13 to 62\% of the cells from the various animals were heavily labeled by \( ^{14}\text{C-leu} \) and showed 10 or more grains in autoradiographs. In contrast, from 4 to 47\% of PPD-stimulated cells were heavily labeled by \( ^3\text{H-U} \), while a maximum of only 4 to 34\% were labeled by \( ^3\text{H-T} \).
Cellular labeling by the three isotopes was minimal during the first 48 to 72 hr of incubation in PPD-stimulated cultures. Maximum labeling occurred near the end of the culture period, from day 4 to day 6. Although the overall response to PPD was generally much smaller than the response to PHA, labeling of tuberculin-stimulated cells in cultures from animals 19 and 48 met or exceeded the levels of $^3$H-T labeling of PHA-stimulated cells on days 5 and 6. Also, $^3$H-U uptake in PPD-cultures from animal no. 19 was the same on day 6 as that in PHA-stimulated cultures, indicating that antigen stimulation of lymphocytes, although delayed, induced a response as strong as that observed in PHA-stimulated cultures.

In most unstimulated control cultures, very little uptake of radiolabel occurred in the DNA, RNA, or protein fractions. Control cultures from animals 19 and 21, however, showed significant grain counts for protein labeling, and lesser amounts for DNA and RNA labeling.

**Morphologic blast determination**

Blast-transformed cells were detected in stained culture smears as large, lightly-stained mononuclear cells whose diameter was approximately 2 to 4 times that of a small lymphocyte (Plate 2, C and D). Nuclear chromatin showed fine structure and occasionally nucleoli were visible (Plate 2, C).

Numerous cells, intermediate in size between the small and large lymphoblasts, were visible throughout the 6-day culture period (Plate 2, B to E) making a strict delineation between blasts and nonblasts difficult. Using an eyepiece micrometer, however, those blast-like cells with a diameter greater than twice that of a small lymphocyte (approximately 12 μ and
larger) were classified as lymphoblasts. The results of morphological determinations in cultures from five animals are presented in Figure 26.

An increase in the number of large blast-transformed cells was observed after day 3 in PHA- and PPD-stimulated lymphocyte cultures. While the number of blast cells continued to rise in most PPD-stimulated cultures until day 6, a decrease in their number was noted in 3 of 5 PHA-treated cultures by that time. Very few lymphoblasts were seen in control cultures throughout the course of the experiments.

Phytohemagglutinin induced a greater degree of blast transformation in stimulated cultures (maximum from 12 to 27%) than did tuberculin PPD (maximum from 2 to 12%). In one PPD-stimulated culture, however, close to 26% of the cells were classified as blasts on day 6 (culture 19).

In most cultures, blast transformation followed cellular uptake of $^3$H-U by about 24 hr. However, blast transformation preceded DNA labeling by at least 12 hr, as shown by photographs C, D, and E in Plate 2. In these photographs, several unlabeled blast cells are shown, although the cells had been incubated with $^3$H-T for 12 hr prior to the slide preparation. During this 12 hr incubation, other blast cells in DNA synthesis took up the isotope and were heavily labeled.

Some lymphoblasts displayed a thick, cytoplasmic uropod (Plate 1, P) which was easily distinguished from uropods seen on small lymphocytes (Plate 1, A to C).

Biochemical sequence of events

On the basis of the lymphocyte responses as determined both by liquid scintillation and by autoradiographic analysis of cellular transformation,
Figure 26. Five graphs which compare the number of large lymphoblasts observed in bovine lymphocyte cultures following stimulation with PHA or PPD. Blast cells were determined as large, lightly stained mononuclear cells with a diameter of approximately 12μ and greater. No less than 200 randomly chosen cells/culture were counted and classified as being either small cells or blast cells. The culture responses are recorded as:

- PHA-treated
- PPD-treated
- control
animal no.:

19

21

25

48

67

(No. of large blast cells per 1000 cells)

(Days following stimulation)
small lymphocyte (approx. 5 to 6 \( \mu \))

large blast cell (>12 \( \mu \))

**Figure 27.** A diagram of the proposed sequence of events which follows in vitro stimulation of bovine peripheral blood lymphocytes by PHA or PPD.

A tentative sequence of biochemical events which occurred after stimulation by phytohemagglutinin or tuberculin was established. The results indicate that this sequence consisted of protein synthesis followed, in turn, by RNA synthesis, histologic blast transformation, and then by DNA synthesis (Figure 27).

The exact relationship between protein and RNA synthesis was not obvious, since conflicting results were found in the liquid scintillation and autoradiographic preparations. By scintillation, RNA synthesis preceded protein synthesis as shown by a greater incorporation of \( ^3 \text{H-}U \) than of \( ^{14} \text{C-} \) leu in the first 24 hr. By autoradiography, however, a greater number of cells were heavily labeled with \( ^{14} \text{C-} \) leu than were labeled with \( ^3 \text{H-}U \) within the first 24 hr. The particular sequence of early events for bovine lymphocytes, as diagrammatically presented in Figure 27, is therefore tentative.
DISCUSSION

Evaluation of culture conditions which affect the \textit{in vitro} reactivity of bovine lymphocytes

Given the proper \textit{in vitro} environment, bovine lymphocytes appear to be able to simulate, within limitations, \textit{in vivo} immunologic responses. The reaction of lymphocytes from a sensitized animal to specific antigen \textit{in vitro} appears to be consistent with a secondary response. Whether or not it can be assumed that the \textit{in vitro} response does parallel, or even in part duplicate, \textit{in vivo} immunologic responses, it is apparent that much information about bovine lymphocyte function can be gained from \textit{in vitro} studies. As bovine lymphocytes have not been routinely investigated previously, it was necessary to define some of the parameters which determined the degree of \textit{in vitro} responsiveness shown by bovine lymphocytes to stimulation by PPD (a specific antigen) and PHA (a nonspecific mitogen). Although the present investigation was not intended to be an exhaustive study of the effects of various sera, cell number, culture duration and stimulant dosage, the results presented do emphasize, however, the important influence which these culture conditions have on lymphocyte responsiveness \textit{in vitro}. Furthermore, some of the results of this investigation raise interesting questions regarding the basic mechanisms involved.

The source and concentration of serum employed in the culture medium, as well as the concentration of cells in each culture, were found to have considerable influence on the degree of transformation observed in bovine lymphocyte cultures. Both newborn calf serum (NBCS) and autologous serum were evaluated at several different concentrations for their effect on the
responsiveness of cultured lymphocytes. Newborn calf serum was found to provide the most optimal environment for bovine cells in those cultures tested and was therefore used throughout the remainder of the investigation.

Those investigators who have worked extensively with cell and tissue culture are aware that the choice of a serum, and its final culture concentration, can be difficult problems to resolve. The question may even be raised as to why serum is required in culture medium at all. There should be valid reasons beyond the fact that it has been used in the past with good success.

Although chemically defined media without serum additions have been examined (Paul, 1970) and do support the growth of a few cell types, optimal media for animal cell growth have invariably contained serum. Considerably improved growth of cells occurred when a few percent of whole or dialysed serum was added to defined media. This serum effect can be attributed to: (1) the great buffering capacity provided by serum, which assures optimal pH in a cellular microenvironment; (2) provision for the proper osmotic pressure required by the cell; (3) the availability of many nutrients which the cell cannot synthesize itself; and (4) the presence of one or more growth factors in the serum, although the nature of such factors is still undefined or in dispute. Tozer and Firt (1964) prepared a defined medium which contained bovine serum albumin, insulin, thyroxine and catalase because they found that a part of the activity of calf serum could be attributed to these factors. They concluded that not only was part of the serum activity supplied by the albumin, but that a second fraction, a macroglobulin (probably an \( \alpha \)-globulin) was also required. According to Paul (1970), the active components of serum are an orosomucoid (an \( \alpha_1 \)-acid
glycoprotein) and an α₂-macroglobulin. Both disappeared from the medium during cultivation of embryonic mouse cells. Each, in isolation, had a small effect in stimulating growth; together they had a marked effect and could replace serum. The macroglobulin could be replaced by nonprotein molecules, such as dextran or Ficoll, but the acid glycoprotein could not be so replaced. These findings seem to confirm many observations which suggest that glycoproteins are the important components in serum.

A fairly standard serum concentration is used by most investigators in cell culture work. Pegrum (1966) stated that the volume of plasma used in human lymphocyte cultures should be between 20 and 33% and Schellekens and Eijsvoogel (1968) were in accord. Ling (1968) recommended 15 to 20% and most workers have employed between 10 and 25% final serum concentrations. With bovine cells, improved growth and in vitro responsiveness of PHA-stimulated lymphocytes were observed when serum concentration was raised in increments from 5% (v/v) to 20% (v/v). This increased in vitro cellular responsiveness of bovine cells as the serum concentration was increased most likely reflected cellular dependence on: (1) a particular serum component; (2) a particular serum effect; or (3) both of these. The lack of enhanced lymphocyte responsiveness in cultures containing more than 20% serum probably reflected the fact that a 20% serum level met all the physical and nutritive requirements of the cultured cells.

Bovine lymphocytes were found to be more responsive to stimulation in medium containing NBCS than autologous serum, even though autologous serum was used at several different concentrations. The reasons for this may be several. Autologous serum does not expose the lymphocytes to any foreign protein capable of inducing transformation (Mills, 1966). There is evidence
that, at least in systems which use other than bovine lymphocytes, calf serum may evoke marked lymphocyte transformation often termed "spontaneous" (Wilson, 1966; Schellekens and Eijsvoogel, 1968; Woodliff and Onesti, 1968). Were there antigens present in the homologous calf serum which might be recognized as "foreign" by the bovine lymphocytes studied, then an increased proliferative response due to antigenic recognition might be expected and observed.

Normal autologous serum from fully-grown animals contains a variety of inhibitory factors which regulate the metabolic activity and proliferation of lymphocytes, the most potent (and the most recently investigated) being \( \alpha \)-globulin (Cooperband et al., 1968). According to Cooperband, allograft rejection and antibody production can be suppressed in animals injected with this \( \alpha \)-globulin. It can prevent the stimulatory effects of both PHA and specific antigen \textit{in vitro}. The mechanism by which a normal plasma protein fraction exerts this immunosuppression, however, is still unknown.

Since \( \alpha \)-globulin has been found to be a potent suppressor and regulator, its presence in autologous, adult serum would explain a lowered \textit{in vitro} response of lymphocytes incubated with this serum. This at first appears to contradict the ideas of Tozer and Pirt (1964) that \( \alpha \)-globulin is partly responsible for improving cellular growth characteristics \textit{in vitro}. If \( \alpha \)-globulins were to attach to the membrane of a lymphocyte in large numbers, this might result in the covering and "hiding" of many or most immunologic receptor sites, a process which would render the cell unresponsive to any stimuli, specific or nonspecific. At the same time, a large number of macroglobulin molecules attached to the cell surface might
serve to strengthen and stabilize the cell membrane to a variety of environmental alterations. Such a "stabilizing effect" is probably the reason why many large molecules are being used as substitutes for serum (in whole or in part) in a variety of synthetic culture media. Coulson and Chalmers (1967b) found that methyl cellulose was suitable as a serum substitute. Bergman et al. (1967) were successful in using dextran in this role, although Kabat and Mayer (1961) stated that dextran was not immunologically inert and Wilson (1966) found that dextran induced lymphocyte transformation. In the results reported here, the addition of dextran to culture medium resulted in a decreased incorporation of isotope in stimulated bovine lymphocyte cultures compared to those without its addition. It is quite possible that this decreased lymphocyte responsiveness might have been the result of the "protective covering" effect just discussed. Such a mechanism of action would not only explain the data obtained but would also lend support to the assumption that large macromolecules, such as Α-globulin, can be immunoregulators.

In addition to specific macroglobulin suppressors, a great variety of immunoglobulins which are not found in newborn calf serum are present in adult serum. Such immunoglobulin could interfere with and make less effective the action of PHA. Also, a number of enzymes and regulatory hormones, not present in NBCS could act to repress a potential lymphocyte response.

Some workers feel that fetuin is an important serum component required in culture systems. Fetuin is a glycoprotein present in high concentration in calf serum. This glycoprotein may be lacking in adult serum and would provide a basis for assuming that NBCS is more nutritionally correct for
cell culture. If Tozer and Firt (1964) are correct, however, α-globulin and fetuin are similar. This would mean that "fetuin" is not strictly a component of NBCS; other causes for greater cellular activity in the presence of NBCS must therefore be sought.

Certainly the nutritive and buffering values of autologous sera were functional in the culture system used in this study since increasing concentrations of autologous sera resulted in increased lymphocyte response. Were NBCS to be exactly the same in buffering and nutritional capacity as autologous sera, the lack of repressor substances in NBCS would be sufficient to explain its increased effectiveness in culture and warrant its continued use as the serum of choice.

Although data to substantiate these positions were not obtained, increased bovine lymphocyte responsiveness in cultures containing NBCS was probably due to (1) lack of repressor substances in NBCS; and (2) small amounts of foreign homologous antigens in NBCS. A fairly high "background" or "spontaneous" isotope incorporation observed in many of the control cultures in this study may be explained by (2) above.

In those bovine lymphocyte cultures which received PHA as a stimulant, an increasing linear relationship was observed between thymidine uptake and the number of lymphocytes in the density range of from 1 to $3 \times 10^6$ cells/ml of culture medium; compared to cultures prepared at $1 \times 10^6$ cells/ml, however, bovine lymphocytes showed a large decrease in thymidine incorporation when they were cultured at only $5 \times 10^5$ cells/ml. The enhancement of DNA synthesis as a result of increasing the number of leukocytes in the original inoculum was therefore interpreted as being a cell-dose effect. It is assumed that increasing the total number of cells in a culture also
increased the number which were capable of responding to stimulation, and this resulted in a higher level of reactivity. Schellekens and Eijsvoogel (1968) and Hughes and Caspary (1970) have observed an enhancing effect of "cell density" on DNA synthesis in cultures of human lymphocytes similar to the effect reported here for bovine lymphocytes. Moorhead et al. (1967) noted that cell density affected the responsiveness of mixed human leukocytes and observed that separation of the two cell populations by a filter membrane prevented DNA synthesis. Moorhead thus suggested that the enhancing effect seen when cell concentrations are increased is the result of a greater opportunity for cell-cell interaction and cooperation. Such an interaction probably results in recruitment of otherwise nonreactive lymphocytes and an overall amplification of the original response. If this mechanism is indeed functional, quite possibly it is mediated via uropods (seen in many of the bovine lymphocyte preparations; Plate 1) and involves both lymphocytes and lymphoblasts. Although data is lacking that a lymphocyte which has interacted with a lymphoblast eventually transforms into a lymphoblast, or that this type of interaction via a uropod is a prerequisite to blast transformation, the evidence so far obtained is consistent with this hypothesis.

It is interesting that DNA-synthesis in antigen-stimulated cultures was also dependent on cell density and showed an optimum responsiveness at $2 \times 10^6$ cells/ml. On the basis of some current concepts of immunologic reactions, one would expect that, in a given cell population, there are a finite number of lymphocytes capable of responding to a given antigen. In an in vitro situation, an antigen such as tuberculin should be ubiquitous in the medium and therefore available to all cells; those that are capable
of responding should transform into blast cells. The fact that a decreased cellular responsiveness was observed at $3 \times 10^6$ cells/ml as compared to $2 \times 10^6$ cells/ml in PPD stimulated cultures must therefore have some explainable basis. The possibility of a very local change in culture pH exerting an unfavorable effect on cellular response seems unlikely since the color of the indicator (phenol red) in the medium did not vary significantly throughout the culture period. Further, in cultures with high cell concentrations, available nutrients are rapidly used up and toxic metabolic products are secreted in high concentration. However, there appears to be no conclusive basis for the assumption that these nutritive conditions were the basis for the decreased response in PPD cultures since cells cultured at the same concentration and stimulated instead with PHA showed very high DNA synthetic activity. The cause for this decreased PPD response at high cell density must therefore lie with the particular way the sensitized cells responded to antigen. It is possible that the antigenically-stimulated cells produced a soluble factor upon transformation which, in high concentrations, caused negative feedback inhibition of its own producer-cell. Such a mechanism would serve, in vivo, to "turn-off" an antibody response after an antigen had been destroyed or removed from the body. This soluble factor could be antibody (Uhr and Møller, 1968), or it could be a specific "inhibitor of DNA synthesis (IDS)" similar to that reported by Smith et al. (1970). At the optimal cell concentration of $2 \times 10^6$ cells/ml of culture medium, then, it might be stated that the two opposing forces (increasing numbers of reactive cells and greater production of feedback inhibitors) are in balance. Such a mechanism of suppression in antigenically-stimulated cultures would imply that either no feedback
factor is synthesized by the PHA-stimulated cells in cultures containing $3 \times 10^6$ cells/ml; or that, if a factor is synthesized, it does not affect the ability of bovine lymphocytes to respond to PHA in the same way that it inhibits the response to a specific antigen. Since a large variety of lymphokines are produced in PHA-stimulated cultures, this latter view is the most likely conclusion.

The change of culture medium in all lymphocyte cultures incubated for more than 3 days was found to be essential to prevent a rapid decrease in culture responsiveness which occurred after the 3rd day of incubation with PHA. When medium was changed on day 3, an unexpected large and rapid increase in isotope incorporation was observed in all PHA-stimulated cultures, whereas cultures whose medium was changed on day 4 exhibited only decreased isotope labeling. This "second-peak" response occurred without the inclusion of mitogen with the fresh medium and exhibited an isotope activity which was almost twice that observed in the earlier peak of isotope incorporation at 48 hr. This secondary enhancement phenomena, which regularly occurred without added PHA or PPD, may be due to any of the following: (a) removal of toxic metabolites from the medium; (b) addition of fresh nutrients to the cells; (c) removal of a proliferation-inhibitor factor (a lymphokine), synthesized by the stimulated cells before the factor could irreversibly inhibit further cellular activation; or (d) a combination (or all) of the above.

The most obvious reason for a decreased culture responsiveness after 3 days of incubation is the buildup of toxic products in the medium. Stanek (1970) stated that if cultures were not transferred to fresh medium within a certain time, degenerative changes would begin which would lead
ultimately to the death of the cellular components. This degenerative process which could be reversed to some extent, would be caused by unsuitable, depleted environmental conditions, such as inadequate nutrition, lack of oxygen, and an increasing concentration of toxic metabolic products. The fact that bovine lymphocytes showed an enhanced responsiveness in the presence of fresh medium lends support to the above suggested basis for the decreased response.

Inhibitor factors were investigated by Smith et al. (1970) and Nilsson (1972) who worked with human lymphocyte cultures. Nilsson found that a marked increase in thymidine uptake occurred when all of the culture medium in PHA-stimulated cultures was removed, either daily or on day 3, and replaced by fresh medium with the same PHA concentration. The same operation in PPD-stimulated cultures, however, resulted in a decreased thymidine uptake.

The results reported by Nilsson for the PHA-response of lymphocytes support the findings reported here for bovine lymphocytes. An important difference, however, is the fact that Nilsson included additional PHA in the fresh medium whereas no additional PHA was included in the medium changes reported here. Further, Nilsson reported that human lymphocytes exhibited a decreased response to PPD after medium change; bovine lymphocyte activity, however, continued to increase in PPD-treated cultures in spite of the change of medium.

The above findings suggest that soluble factor(s), formed by PHA-stimulated lymphocytes, are of great importance in determining the duration and degree of cellular responses in vitro. It seems improbable that the sharp increase in radioisotope uptake after medium change in PHA cultures
is an artifact, caused by manipulation of the cultures, because (1) the effect was not observed when the medium was changed on day 4, and (2) the effect in similarly-treated PPD-cultures was not as marked.

The nature of the factor(s) involved was not resolved by the present study. The finding that RNA and protein labeling were strongly affected, in addition to DNA-labeling, argues against the involvement of just a DNA-inhibitor factor. If any lymphokine is involved at all, a "transformation-" or "proliferation-inhibitor" factor is more probable. Additional experiments to determine the nature of this "second-response" phenomenon were not performed but would be a most interesting area for future investigation.

Transformation of bovine lymphocytes by PHA and PPD was found to be dependent on the dose of stimulant used. Each stimulant exhibited an optimal growth-enhancing concentration; below and above this optimal concentration the response to either was diminished. This type of a relationship between the dose of PHA or PPD and the lymphocyte response is in agreement with studies of lymphocytes from other species such as man (Caron, 1966; Richter and Naspitz, 1967; Naspitz and Richter, 1968); guinea pig (Phillips and Zweiman, 1970); and hamster (Fernald and Metzgar, 1971). Caron (1967) noted that there was an inhibition of transformation at high antigen concentrations similar to the observed prozone of inhibition of precipitation in the zone of antigen excess.

The increase in culture responsiveness as the stimulant concentration increased in both PHA and PPD cultures may be explained by assuming that more cells (and perhaps more receptor sites on an individual cell) were in contact with the stimulating agent as its concentration increased. Maximum
response in either system occurred when all available receptors on all cells capable of a response to that agent had just become saturated. It might be expected then, that increasing the stimulant concentration beyond this optimal level should have no additional effect on the cultures. However, an inhibitory effect was observed when high doses of either antigen or mitogen were used. This implied that there might be a common (or a similar) mechanism to this inhibitory effect.

The production of a variety of soluble lymphocyte factors has been discussed. It is quite probable that stimulated bovine lymphocytes are synthesizing factors in response to in vitro activation; these factors may be growth promoting or cytotoxic in action. Negative-feedback growth regulators, however, or cytotoxic factors, may be produced only when the stimulating substance is present in large quantities, while the culture stimulants and growth-enhancing factors may be the major influencing factors involved in cell response at lower concentrations of PHA or PPD. The optimal PHA or PPD concentration would then be that concentration sufficient to induce maximum cellular responsiveness and production of enhancing factors while stimulating limited or no production of negative-feedback inhibitors (either specific immunoglobulins or nonspecific lymphokines).

Perhaps the basis for the observed dose-dependent effect is more structurally oriented. In this regard, the inhibitory effect of high PHA concentration is an interesting observation. One might think that the strong cell agglutination which occurs at high PHA concentrations would enhance cellular responsiveness by increasing the probability of cell-cell contacts. As this enhancing effect is not found to occur at greater-than-optimal doses of PHA, the excess agglutination of cells may actually hinder
or prevent the entrance of thymidine into stimulated cells, thereby masking the actual occurrence of cell transformation. Alternatively, the dose-effect may be directly related to the mechanism of PHA-action itself. It is thought that the rapid stimulation of cells by PHA is a result of altered permeability of cellular membranes, especially those of the intra-cellular lysosomes. At low levels of PHA, quantities of lysosomal enzymes are released which move to the nucleus to selectively destroy histones and derepress nuclear DNA, with a subsequent increase in the rate of DNA transcription and replication (Rubin et al., 1970). One might then conclude that an excess of PHA in the cellular environment could cause the release of an excess of internal lysosomal digestive enzymes with a consequent cytotoxic effect and decreased culture responsiveness.

The results of this investigation imply that the mechanism of stimulation of bovine lymphocytes by PPD is different than that of PHA. The observed depression of lymphocyte response at high PPD levels may therefore have an immunologic basis which is very different from that described for PHA above. Sensitized bovine lymphocytes were observed to respond to stimulation by low levels of PPD at a much slower rate than they did to stimulation by PHA in an identical culture arrangement. This reflected the fact that only those cells which are immunologically capable of responding to PPD will do so in vitro, and this may be only a small population in the original cell culture. According to Marshall et al. (1969), only 2% of the starting population in a lymphocyte culture of sensitized cells are capable of responding to PPD stimulation compared to 50 to 70% which respond to PHA. Most of the blast cells which appear on days 5 and 6 in PPD-stimulated cultures are thus thought to derive from this small population by repeated
mitosis, a process which begins slowly due to the small number of sensitive cells but which increases at an exponential rate.

On the basis of several lines of evidence, it is likely that the type of in vitro response of bovine lymphocytes to tuberculin PPD is distinct from the response to PHA, and represents antigen-specific cellular reactivity: (a) tuberculin-induced lymphocyte proliferation in this study was limited to cells from tuberculin-sensitive animals, with no cellular proliferation observed when lymphocytes of nonsensitive animals were incubated with the same concentrations of PPD; (b) the lymphocytes of tuberculin-sensitized animals were no more responsive to PHA than were the cells of tuberculin-nonsensitive animals; (c) PPD-stimulated lymphocytes exhibited, during the 6-day culture period, a consistent pattern of responses which were different and distinct from those induced by PHA; and (d) lymphocytes from sensitized animals showed a similar range of responsiveness in dose-dependence experiments.

The molecular mechanisms leading to cellular response following PPD stimulation are not really known but they may be related to antigenic alteration of surface charge or modification of membrane receptor molecules. After antigen "capture" or "recognition", PPD may be processed or passed on to another cell in a cooperative immune response to the antigen. All this takes time and may help to explain the in vitro delay in the response of bovine lymphocytes to PPD. When large doses of PPD are present, however, an immunological "paralysis" may occur due to a large and immediate feedback inhibition by immunoglobulin molecules or lymphokine inhibitor factors. Such an action occurs in vivo (Mitchison, 1964) and high-dose effects may be its correlate in vitro.
A possible mechanism for the high-dose inhibition of cellular response involves a modification of cellular membrane structure after an overloading of receptor sites. Such a modification might lead to one of several interesting results: (1) a reduced membrane permeability which might prevent a cellular response due to insufficient metabolic activity; or (2) an altered surface antigenic structure, which might result in loss of cellular recognition by mutually-cooperating cells and, possibly, suppression or even cytotoxic action by one cell against the antigenically-altered cell (Gershon et al., 1972).

Very little data is available, either in this investigation or from similar studies reported in the literature, on which to base an adequate statement of the mechanism of inhibition of high doses of lymphocyte stimulants. Immunologic phenomenon, involving feedback mechanisms, soluble mediators, and in vitro cell-cell repression or destruction would appear to form the most likely basis for inhibition at the present time.

The maximum number of large blast cells observed in PHA or PPD stimulated cultures ranged from 12 to 27% of all those cells randomly counted on slide preparations made from those cultures. In both PHA- and PPD-stimulated cultures, these peaks in morphologic blast formation occurred late in the culture period, particularly during the 3-day incubation period following the change in culture medium on day 3. Whether the introduction of fresh medium had any influence on this morphologic blast formation is not certain; such an action can probably be ruled out, however, because unstimulated control cultures showed no increase in the number of blast cells following the change of medium.

The number of blasts observed in stimulated bovine cultures is low.
Phillips and Zweiman (1970) report that up to 90% of the guinea pig lymphocytes were transformed by day 4 in either PHA- or PPD-stimulated cultures. Ling (1968) reported that only a maximum of 39% of rabbit lymphocytes are blast-transformed by day 3, but Sell et al. (1965) found up to 80% blasts in their cultures in the same period of time. The reasons for such a low percentage in blast formation by stimulated bovine lymphocyte cultures is uncertain at the present time. No doubt, much of the variation between reported levels of blast formation in different species is due to an investigator's own definition and restrictions as to what he will, in fact, call a blast cell. To many workers in the field, any mononuclear cell which is larger than a small lymphocyte and has a lightly-staining nucleus may be included in that class of cells called "blasts". In the bovine system, however, a heterogeneous distribution in cell sizes is normally present even in unstimulated cultures. According to Schalm (1965), a single film of bovine blood will show all gradations from small to large lymphocytes, whereas blood smears made from other animals such as horse or swine exhibit cells more uniform in size. This heterogeneous distribution in bovine lymphocytes has made the definition and detection of blast cells in stimulated cultures quite difficult. A rather large dimension of approximately twice the diameter of an unstimulated small bovine lymphocyte was therefore established as the minimum size for a blast cell so that many "medium-sized" lymphocytes, often seen in the bovine cultures, would not be placed in the "blast" classification. The resulting low percentage of blast forms in bovine cultures should not be interpreted, however, as reflecting a low in vitro responsiveness to stimulation by PHA or PPD, since isotope incorporation studies indicate that the in vitro response of bovine lymphocytes.
is strong and compares well to the data reported for other species.

Evaluation of isotope incorporation

The use of two different isotopes ($^3$H and $^{14}$C) as tags on the precursor molecules used in this study posed some difficult questions in regard to data interpretation, since the specific activity of the two isotopes was so different. Compared to $^{14}$C, the radioactive half-life of tritium ($^3$H) is very short: only 12.3 years, as contrasted to the 5,760-year half-life for the $^{14}$C isotope (Rogers, 1967). For the same number of isotopic atoms, therefore, tritium has many more disintegrations/unit time (due to its shorter half-life) than does carbon-14. The advantage of tritium labeling lies in the fact that higher specific activities can be obtained than with carbon-14; carrier-free tritium has an activity of 57.5 Ci/milliatom, whereas carbon-14 has only 0.054 Ci/milliatom (Wolf, 1964). In a similar manner, if a sample of carbon-14 is said to have 1 uCi of radioactivity ($1 \mu\text{Ci} = 2.2 \times 10^6 \text{ disintegrations/min}$), it would have to contain many more isotopic atoms than a sample of tritium with the same amount of radioactivity. This means that a determination of the amount of radioactivity incorporated by a cell or cell culture does not define the number of isotopic molecules taken up by that sample, especially when two different isotopes with very different half-lives are compared.

For these reasons, the comparison of RNA, DNA, and protein synthesis in FHA- or PPD-stimulated bovine lymphocyte cultures, on the basis of counts-per-minute of labeled precursors incorporated, does not appear to be completely valid. A more satisfactory comparison would be based on the approximate numbers of radiolabeled molecules taken up into the RNA, DNA,
and protein of the stimulated cells. To do this, it is necessary to calculate the approximate number of molecules of radiolabeled precursors which were added to each culture at the time of pulse-labeling. In the experiments reported here, 1 μCi each of $^3$H-T and $^3$H-U, and 0.25 μCi of $^{14}$C, were added to separate duplicate cultures. As mentioned earlier, the specific activity of $^3$H-U used was 14 Ci/mmol; that of $^3$H-T was 6.7 Ci/mmol; and that of $^{14}$C-leu was 262 mCi/mmol. Since 1 mole of any substance contains $6.025 \times 10^{23}$ molecules (Moore, 1962), 1 millimole contains $6.025 \times 10^{20}$ molecules.

By calculation, then, since the $^{14}$C-leu used in this study had a specific activity of $2.62 \times 10^5$ μCi/$6.025 \times 10^{20}$ molecules, 0.25 μCi (the amount added per culture) would be associated with $57.5 \times 10^{13}$ molecules. Similar calculations indicate that, for $^3$H-T, 1 μCi of specific activity is associated with $9 \times 10^{13}$ molecules, while 1 μCi of specific activity is associated with $4.3 \times 10^{13}$ molecules of the $^3$H-U used.

A satisfactory comparison of the incorporation of radiolabel into activated cells can now be made by calculating what the recorded activity, in cpm, will be for the same number of molecules of each labeled precursor. If $10^{13}$ molecules of each precursor is incorporated, the following approximate activity will be recorded: for $^{14}$C-leu, 9,565 cpm; for $^3$H-T, 244,444 cpm; and for $^3$H-U, 511,628 cpm.

Figure 28 presents the averaged response of lymphocyte cultures from seven animals as determined by liquid scintillation and recorded in cpm/$10^6$ cells. The lines on the graph indicate that the incorporation of $^3$H-U was greater than that of either $^{14}$C-leu or $^3$H-T at 6 hr after initial stimulation of cultures. Further, the measured activity of $^{14}$C-leu was found to be significantly less after day 1 than the activity detected for $^3$H-U.
Figure 28. A graph of the *in vitro* relationships between $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporation by PHA- and PPD-stimulated cultures: an averaged response using pooled data from the seven animals tested. The culture responses are recorded as follows:

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Averaged response of all animals; pooled data
or $^3$H-T. When the measured activity in cpm for each isotope is converted to the number of molecules incorporated into the cells, however, a very different relationship is observed, as is presented in Figure 29. Both in PHA- and in PPD-stimulated cultures, the number of $^{14}$C-leu molecules incorporated was far greater than the number of $^3$H-T or $^3$H-U molecules incorporated. As early as 6 hr after initial stimulation, leucine incorporation exceeded uridine uptake by as much as 24 times in PHA-stimulated cells, while it exceeded uridine uptake by more than 32 times in PPD-stimulated cultures in the same period of time. Although a greater number of uridine molecules than thymidine were incorporated during the first 24 hr, the uptake of uridine was considerably less than of either leucine or thymidine for the remainder of the culture period. Such evidence lends support to the autoradiographic results presented earlier which indicated that the overall leucine incorporation was greater than uridine or thymidine incorporation in both PHA- and PPD-stimulated cells.

A question may be raised in regard to the validity of the autoradiographic data which employed two different isotopes, since it is known that carbon-14 emits a more energetic $\beta$-ray (155 keV) than does tritium (19 keV). According to Wolf (1964), the maximum energy of a $\beta$-ray from tritium is so low that the particle's range in the overlying photographic emulsion is only about 2 $\mu$. Over 90% of the radiation energy for this particle is released in a sphere of 0.5 $\mu$-radius. This is so small that for each molecule containing $^3$H atoms, only one or two grains of silver salt will be exposed. With carbon-14, however, the higher energy $\beta$-ray exposes many more silver grains of the photographic emulsion with each disintegration. In regard to a measurable efficiency, then, Rogers (1967) stated that,
Figure 29. A graph of the *in vitro* relationship between the number of molecules of $^3$H-thymidine, $^3$H-uridine, and $^{14}$C-leucine incorporated /$10^6$ stimulated lymphocytes: an averaged response for the seven animals tested. The points on the graph were obtained by converting the corrected counting rates of Fig. 28 into the no. of molecules of each isotope which would have to be incorporated to achieve that particular isotopic counting rate. The culture responses are recorded as follows:

**PHA-stimulated cultures**
- DNA —○—
- RNA —△—
- protein —□—

**PPD-stimulated cultures**
- DNA —•—
- RNA —△—
- protein —□—
Averaged response of all animals; pooled data
with a smear of isolated, flattened cells and an emulsion layer 1 to 2 μ thick, one might expect about 5 silver grains per 100 disintegrations for tritium and 40 to 45 grains with carbon 14. For the same number of disintegrations, then, carbon-14 will cause the formation of approximately 8 to 9 times more silver grains than will tritium. However, due to the higher specific activity of the particular tritium isotopes employed in this study, the same number of disintegrations for each labeled precursor requires approximately 25 times more $^{14}$C-leu than $^3$H-T and approximately 50 times more $^{14}$C-leu than $^3$H-U. Thus, for a cell to be labeled with 10 grains or more, the following approximate numbers of molecules for each isotope would need to be incorporated: for $^{14}$C-leu, $28.8 \times 10^9$ molecules; for $^3$H-T, $8.4 \times 10^9$ molecules; and for $^3$H-U, $3.9 \times 10^9$ molecules. The qualitative relationships expressed in Figures 21 to 25 are therefore valid.

The incorporation of these labeled precursors into DNA, RNA, or protein molecules is a useful tool for studying in vitro cellular responses to antigenic or mitogenic stimulation. However, the assumption that the amount of incorporated precursor is proportional to the amount of newly synthesized DNA, RNA, or protein may not always be correct. Since radioactive precursors enter intracellular pools, fluctuations in these pools can enhance or reduce incorporation rates without corresponding changes in the actual rate of synthesis of the molecule being studied.

This problem of intracellular pools is probably more critical when isotope incorporation is evaluated by autoradiography than when incorporation is evaluated by liquid scintillation. Since all intracellular soluble pools are present in the whole cells mounted on autoradiographic slides, any isotopically-labeled molecule stored in an intracellular precursor
"pool" rather than in a newly synthesized molecule will still be able to induce grain formation in the overlying nuclear emulsion. In cells with large intracellular pools for DNA, RNA, and/or protein precursors, this could lead to the formation of a great number of autoradiographic grains unrelated to the molecular synthesis being evaluated. In samples prepared for liquid scintillation, however, all cells were ruptured, and most free and soluble molecules were dispersed, leaving only acid-insoluble, precipitated cellular material to be evaluated.

For example, in autoradiographs prepared from PHA-stimulated cells of animal no. 21 on day 5 of culture, grain counts alone indicate that for every 10 molecules of \(^3\)H-T incorporated into a cell, approximately 9 molecules of \(^3\)H-U are taken up, while over 130 molecules of \(^14\)C-leu are incorporated. By liquid scintillation analysis of the same cultures, however, this relationship is somewhat different; for every 10 molecules of \(^3\)H-T, 0.9 molecules of \(^3\)H-U and only 100 molecules of \(^14\)C-leu are incorporated into cellular precipitates. Although both methods indicate that protein synthesis on day 5 exceeds either DNA or RNA synthesis, the much higher numbers of uridine and leucine molecules detected by autoradiography are an indication of the relative sensitivity of this technique to intracellular pools. Consequently, one can more confidently state that isotope incorporation as measured by liquid scintillation more accurately reflects the amount of actual molecular synthesis being performed by a cell or cell culture.

In conclusion, the quantitative molecular relationships just evaluated confirm the proposed model for the sequence of events, presented in Figure 27, which occurs after in vitro stimulation of bovine lymphocytes by PHA or PPD.
The in vitro response of bovine peripheral blood lymphocytes to stimulation by phytohemagglutinin (PHA-M) and tuberculin (PPD) was evaluated using, at different times, cells from 21 head of cattle. A variety of culture conditions were studied and found to significantly effect the ability of the lymphocytes to incorporate radiolabeled metabolic precursor molecules following mitogenic or antigenic stimulation in vitro. DNA synthesis in activated cells was evaluated by cellular uptake of $^{3}H$-thymidine; RNA synthesis was evaluated by the uptake of $^{3}H$-uridine, and protein synthesis was evaluated by incorporation of $^{14}C$-leucine.

Newborn calf serum provided a more suitable culture environment for bovine lymphocytes than did autologous serum employed at the same concentration. The optimal serum concentration was 20% (v/v). The use of dextran (10%, v/v) as a serum substitute was found to provide no cultural advantage.

Lymphocytes were routinely cultured at from 1 to $2 \times 10^{6}$ cells/ml of culture medium since maximum isotope incorporation occurred at this cell concentration. Cells from all test animals demonstrated a strong response to stimulation by PHA at a final culture concentration of 10 µl PHA-M/ml; lymphocytes from tuberculin-sensitized animals responded well to stimulation by PPD at a final concentration of 10 µg PPD/ml of culture medium. Mitogen or antigen doses above or below these concentrations resulted in a decreased level of isotope incorporation. Two major peaks of lymphocyte response to PHA were observed: the first occurred at 48 hr after initial stimulation, and the second occurred at 96 to 120 hr when culture medium was changed on day 3. No secondary peak in isotope incorporation occurred
if medium was changed on day 4. This dependence of lymphocytes in PHA-stimulated cultures on fresh medium for continued in vitro responsiveness may have reflected the buildup in the culture medium of a proliferation-inhibition factor whose action was reversible if removed prior to the 4th day of culture.

Lymphocytes from tuberculin-sensitized animals responded slowly to PPD-stimulation in vitro, but by day 6 of culture the incorporation of all three isotopes used for metabolic evaluation approached or exceeded the levels of activity found in similarly-treated PHA-stimulated cultures. Lymphocytes from tuberculin-nonsensitive animals did not respond in vitro to any concentration of PPD. Whether cells were from tuberculin-sensitive or nonsensitive animals had no effect on their ability to respond to PHA in vitro.

From 20 to 30% of the cells in PHA- and PPD-stimulated cultures responded with morphologic blast transformation as shown by a large cell size (approximately 12 μ diameter and larger), and a large, lightly-stained nucleus which exhibited fine chromatin structure and an occasional nucleolus. Uropod formation was observed in most stimulated cultures and probably reflected the means of intercellular communication employed by stimulated cells to "recruit" nonstimulated cells.

By means of liquid scintillation and autoradiography, the relative amounts of 3H-thymidine, 3H-uridine and 14C-leucine incorporated into DNA, RNA, and protein were evaluated. The results indicated that the following sequence of metabolic events occurred after PHA or PPD stimulation of bovine peripheral blood lymphocytes: protein synthesis, RNA synthesis, morphologic blast transformation, and DNA synthesis.
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