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THE EFFECTS OF PROTEASE INHIBITORS ON LYMPHOCYTE TRANSFORMATION

BY

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A B S T R A C T

This work presents an investigation of the effects of various protease inhibitors but mainly soybean trypsin inhibitor (SBI) on transformation of human and mouse lymphocytes induced by a variety of agents. SBI was found to inhibit transformation of human lymphocytes induced by mitogens (leucoagglutinin, concanavalin A, $NaIO_4$, phytohemagglutinin, pokeweed mitogen) or in the mixed lymphocyte reaction. Other protein protease inhibitors which were effective were lima bean trypsin inhibitor (LBI) and ovomucoid (OM). Synthetic protease inhibitors which were effective were N-tosyl-L-Lysine-chloromethyl-ketone (TLCK), tosyl-phenylalanyl-chloromethyl ketone (TPCK), N-tosyl-L-argine methyl ester (TAME), phenyl-methylsulfonylfluoride (PMSF) and ε-aminocaproic acid (EACA). Exposure of the cultures to the inhibitors did not affect the viability of the cells. SBI covalently cross-linked to sepharose beads also inhibited the MLR and mitogen stimulation. Kinetic studies, done with human and mouse cells, showed that the inhibitor acted early after the addition of the mitogen or the mixing of the allogenic cells in the MLR. The effect of the plasma and of other cell types present in the cultures on the degree of inhibition was also investigated. The data shown support the hypothesis that protease action at a cell surface is an essential early event common to all types of lymphocyte transformation.

R E S U M E

J'ai voulu, dans ce travail, examiner l'effet de plusieurs inhibiteurs trypsiques mais surtout de l'inhibiteur trypsique du soja (SBI) sur la transformation des lymphocytes humains et de souris induite par toute une variété d'agents différents. Ainsi, il fut démontré que le SBI cause une profonde inhibition de la transformation lymphocytaire induite par les mitogènes (leucoagglutinin, concanavalin A, $NaIO_4$, phytohemagglutinin, Pokeweed mitogen) ou dans la réaction lymphocytaire mixte (MLR). En même temps, l'inhibition est causée par divers autres inhibiteurs protéiques de protéases (LBI et OM) ou divers inhibiteurs synthétiques de protéases tels que: N-tosyl-L-Lysine-chloromethyl-ketone (TLCK), tosyl-phenylalanyl-chloromethyl ketone (TPCK), N-tosyl-L-arginine methyl ester (TAME), phenyl-methylsulfonylfluoride (PMSF), ε-aminocaproic acid (EACA). La viabilité des cellules ne fut aucunement affectée par une longue exposition des cultures aux inhibiteurs. Lié de façon covalente au Sepharose, le SBI inhibe la MLR et l'activité des mitogènes presque complètement. Des études de cinétique faites avec des cellules humaines et de souris ont démontré que les inhibiteurs agissent tôt après l'addition des mitogènes ou le mélange des cellules allogènes dans la MLR. Enfin, le rôle joué par le plasma et par d'autres types de cellules présentes dans les cultures fut aussi étudié.

Toutes les données confirment l'hypothèse que l'action protéasique à la surface cellulaire est une étape précoce essentielle et commune à tous les types de transformation lymphocytaire.

R E M E R C I E M E N T S

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

I. GENERAL FEATURES

A. Purpose of this Research

It was over 15 years ago that human peripheral blood lymphocytes started to stir some interest among a few scientists. Indeed Hungerford *et al* (1) and Nowell (2) were the first to describe clearly what seemed a very curious phenomenon: cells from peripheral blood that were cultured in medium usually showed no sign of cellular division, except when a small quantity of Phytohaemagglutinin (PHA), an extract of the red kidney bean, *Phaseolus vulgaris*, was added to the culture. Then, the morphology of some of the cultured cells was greatly changed and the resting cells became very active. It was subsequently shown by Carstair (3,4) that it was the small lymphocytes that were being "activated" or "stimulated", a phenomenon now known as blastogenesis, due to the formation of larger blast cells which is an obligatory stage prior to DNA synthesis and cell division.

Since then, a large variety of agents has been added to cultured blood cells in order to cause stimulation and consequently, there is today a large list of compounds that are known to cause blastogenesis. However, if lymphocyte transformation is now being intensively used by people from

all branches (cell biologists, immunologists, histologists, geneticists, molecular biologists, biochemists, etc.) the actual mode of action of the different stimulating agents is not yet known. The purpose of this work is to shed some light on the mechanism of action of at least some stimulating agents.

One of these stimulating agents (the others will be discussed later in this work) is trypsin. In 1974, Visher (5) and Kaplan and Bona (6) showed that a tryptic treatment of mouse spleen lymphocytes caused blastogenesis. Kaplan and Bona hypothesized that the trypsin acted by short-circuiting an endogenous protease whose action was supposed to be essential to the subsequent events of the blast transformation.

As a test of this hypothesis, I have studied the effects of protease inhibitors, both protein: soybean trypsin inhibitor (SBI), lima bean trypsin inhibitor (LBI), ovomucoid (OM) and synthetic: N-tosyl-L-Lysine-chloromethyl ketone (TLCK), tosyl-phenylalanyl-chloromethyl ketone (TPCK), N-tosyl-L-arginine methyl ester (TAME), phenylmethylsulfonylfluoride (PMSF), ε-aminocaproic acid (EACA) on the blastogenesis, in human peripheral blood lymphocytes and mouse spleen lymphocytes caused by a large battery of stimulating agents. The hypothesis predicts that blastogenesis should be sensitive to inhibition by anti-proteases.

B. Cellular Components of Mammalian Blood

Human peripheral blood is commonly divided into two major parts: (1) the red blood cells or erythrocytes; and (2) the leukocytes blood platelets and plasma. The leukocytes can be further subdivided into two major constituents: the granulocytes or polymorphonuclear leucocytes and the agranulocytes which include the lymphocytes and the monocytes. It is towards the latter part that the attention was directed.

1) Lymphocytes

As a result of the new interest shown in lymphocytes more than a decade ago and of the multitude of experiments that have followed, it became apparent that the lymphocyte was the keystone of the group of cells involved in immunological reaction. The common lymphocytes (also called small lymphocyte) is the smallest of the five kinds of leukocytes. The cell is small in size since it contains little cytoplasm and has a very small nucleus with the nuclear chromatin in a very condensed form. Next to the neutrophils, the lymphocytes are the commonest leukocytes seen in normal blood. They make up about 25% of the leukocyte population of the human blood but their total number can easily vary (20%-30%) from day to day.

A living lymphocyte may seem at first glance to be a very active cell. They move about at great rates, approaching

other kinds of cells, infiltrating themselves between other cells and even entering and passing right through the cytoplasm of some cells. Indeed, lymphocytes can migrate right through the cytoplasm of endothelial cells to get out of a capillary.

In their cytoplasm on the other hand, only a few mitochondria are present suggesting that they have a very low metabolic rate. The chromatin of their nucleus is so condensed that it is hard to envisage much transcription occurring. They must therefore have a very low basal DNA and RNA synthesis, as shown by studies of incorporation of labelled precursors. Only a few free and attached ribosomes are seen, suggesting that there must be very little protein synthesis normally occurring.

It seems, therefore, that there is a discrepancy between the behaviour of the lymphocytes <u>in vivo</u> and the resting state of their cytoplasm, which gives the impression that lymphocytes play no active role in the immunological reaction. However, it was said by A.W. Ham: "Lymphocytes are important, not because of what they are, but because of what they become". (7)

Lymphocytes can be subdivided according to a variety of different criteria including: their surface antigens, reaction to different stimuli, life span and migratory habits. It is now accepted that the small lymphocytes fall under two categories: the "T" lymphocytes and the "B" lymphocytes.

The first type, the T lymphocytes, is processed through the thymus and is mainly concerned with cellular immunity. The B lymphocytes, on the other hand, are thought to be processed in the chicken, through the Bursa of Fabricius and to have the capacity to differentiate into plasma cells, by giving immunocompetence to cells that will subsequently produce antibodies. The two sub-classes of lymphocytes derive their name from the two main organs of the chicken lymphoid system; the thymus, made essentially of epithelial cells and where a large number of lymphocytes are seen and the Bursa of Fabricius, an organ found on the small intestine. There is no Bursa of Fabricius in mammals, but possible candidates for a functional equivalent include Peyer's patches, tonsils and other lymphoid tissues.

In vitro, the T and the B lymphocytes can be separated on the basis of physical characteristics: charge, density, etc. The two main populations of lymphocytes can also be distinguished by looking at the antigenic determinants on one or both populations or by looking at their capacity to respond to different stimulants. Regarding the surface markers on the T and B lymphocytes, one of the main characteristics of the T lymphocyte is its ability to form rosettes with sheep's red cells. Only human and pig lymphocytes appear to have this property although a related

phenomenon has been described in mouse (8). As for the B lymphocytes, the presence of surface immunoglobulin is the major specific determinant on the membrane.

In humans, approximately 50% to 80% of the peripheral lymphocytes form a rosette with sheep red cells indicating that they are T lymphocytes and only about 20% to 30% of the peripheral cells give a positive reaction to the immunofluorescent test for the immunoglobulin, therefore indicating that they are B lymphocytes.

2) Blast Transformation

The first noticeable changes that occur after the transformation of lymphocytes by different agents are seen at the membrane level. Besides some changes in the electrical resistance of the membrane (9) Quastel and Kaplan (10) have shown that there was a net increase in the K^{+} ion transport 5 to 30 minutes after stimulation while Whitney and Sutherland (11) reported similar observations regarding the Ca^{++} ion. The role of the ion fluxes in the blastogenesis has been reviewed recently by Kaplan (12). The membrane enzymes adenyl cyclase (13) and Na and K ATPase (14) activity was also shown to increase very soon after the stimulation. Other events occurring early in blastogenesis include an increase in protein synthesis, in RNA synthesis, in DNA synthesis and in carbohydrate metabolism. A variety of changes also occurs

in the ultrastructure of the transformed cell; the cytoplasm becomes loaded with free and aggregated ribosomes; the nucleus has most of its heterochromatin replaced by euchromatin, which suggest that it enters in a more active state and a large number of mitochondria are now seen in the cytoplasm. The resting cells, after the appropriate stimulus, are completely transformed to a very active state and are now able to complete their particular function.

3) Monocytes

The monocytes constitute about 5% to 10% of the normal leukocytes population. Unlike the lymphocytes, they have a relatively abundant cytoplasm and a well developed horseshoe shaped nucleus.

The macrophages seem to have a very specific role in the immune response. Indeed, pure population of lymphocytes containing no phagocytic cells, show a reduced blastogenic and antibody response to specific antigens *in vitro*. Addition of macrophages restores this response to normal level. This tends to indicate that the macrophages are needed for the normal response to antigen stimulation (15,16). It was also observed that human monocytes exposed to antigens *in vitro* and washed free of extracellular antigens can induce transformation of autologous lymphocytes (17).

Antigens are not the sole agents capable of demonstrating the active role of the macrophage in the immune response. Alter and Bach (18) showed the essential role played by the macrophages in the mixed lymphocyte transformation (MLR), while Rosenstreich and collaborators (19) showed the absolute macrophage dependency of the T lymphocyte activation by mitogens.

Therefore, considering the monocyte's important role in the lymphocyte stimulation and keeping in view Kaplan and Bona's hypothesis (6), the large number of lysosomes present in the monocytes then becomes important. Indeed, lysosomes were shown to contain a multitude of enzymes including Cathepsin D, the major acid hydrolase present, and Cathepsin B; the only well characterized human thiol proteinase. Recently, Rojas-Espinosa and collaborators also isolated from macrophages a lysosomal chymotrypsin-like esterase (20). Since it cannot clearly be stated, according to Kaplan and Bona's hypothesis, whether the site of action of the hypothesized enzyme(s) is on the lymphocytes itself or on the macrophages, or even if the enzyme(s) are produced by the lymphocytes or by the macrophages, then, the presence and the role of the lysosomal enzymes in macrophages takes a new dimension and will therefore be discussed later in this work.

<u>En résumé</u>, macrophages are certainly good candidates for the production of the hypothesized enzyme(s) and this is one of the question I tried to elucidate in this work.

C. Description of the Different Agents Causing Lymphocytes Transformation

Since a large variety of agents were used to study the molecular events of the lymphocyte blast transformation, it is important to know their different characteristics and compare their different mode of action.

1) The Mitogens

The mitogens are without any doubt the most popular agents used in the triggering of blastogenesis in lymphocytes. This is largely due to the fact that mitogens, depending on the dose and on the type of cells, can cause up to 80% of the total lymphocytes to undergo transformation in culture. Some of the most common mitogens used include PHA, Concanavalin A (ConA), Pokeweed mitogen (PWM), sodium periodate (IO_4^-) and Leucoagglutinin (LAG). These agents although they transformed the majority of the cells present, show some specificity by stimulating only a specific type of cell (ex. PHA and ConA are known to stimulate only the T cells while PWM can stimulate both T and B cells. In mouse, Lipopolysaccharide (LPS) only stimulates the B lymphocytes).

(a) PHA

PHA is an extract of the red kidney bean Phaseolus vulgaris. This extract has a dual role: it can agglutinate erythrocytes and it can also activate lymphocytes, although the agglutinating ability or

property is not essential to the mitogenic activity of the extract. Regarding some of its physical aspects, it is known that PHA has a M.W. of 115,000 to 140,000, and that it contains Ca^{++} and Mn^{++} ions (21). Its concentration in culture is, as for other mitogens, critical. PHA when put in culture is capable of precipitating several fractions of human serum including the α_2 macroglobulin and the IgM immunoglobulin. We also found in our laboratory that certain preparations of PHA (PHA-M Difco) show a small but significant proteolytic activity.

(b) Con A

Con A is the best characterized of the plant lectins. Isolated from the jack bean Canavalia ensiformis, it is known to bind specifically to a D-glucopyranoside and a-D-mannopyranoside and to polysaccharides of glycoproteins containing such residues (22). At pH 7.0 to 7.5 Con A occurs mainly as a tetramer of subunits of molecular weight of 25,000 each, but if the pH is lowered to 6.0, it is found that the molecule is in the form of a dimer. Unlike most of the plant lectins, Con A is a pure protein and does not contain any carbohydrate. In our laboratory, Con A-stimulated synthesis of DNA in human cells was shown to peak on the third day and on the second day when mouse cells were cultured.

(c) PWM

The pokeweed mitogen extracted from Phytolacca americana is different from PHA and Con A because of its ability to stimulate both T and B lymphocytes in the mouse and in man. It has a M.W. of about 32,000 and appears to be a single glycoprotein chain.

(d) LAG

Leucoagglutinin is a glycoprotein isolated, as PHA, from the kidney bean Phaseolus vulgaris. The preparation has the advantage of being free of both erythroagglutinating and mixed agglutinating properties shown by the PHA preparation. LAG is considered to be a tetramer made of identical subunits, each having a M.W. of 31,000 (23). The total M.W. of the glycoprotein determined by ultracentrifugation is 126,000. It was shown that LAG does not have to be internalized to stimulate lymphocytes (24). Therefore, its high degree of purity, its homogeneity and its lack of erythro-agglutinability and mixed agglutinability properties make LAG one of the best agents known to cause lymphocyte stimulation.

(e) Sodium Periodate

Human lymphocytes can also be activated by incubation with IO_4^- (25). It was thought by Novogrodsky et al (25) that the IO_4^- acted by oxidizing the surface

sialoglycoproteins and by forming aldehyde groups which, it was postulated, interacted with the amino groups on the cell surface with the resulting cross-linking causing the initiation of the transformation. However, O'Brien et al in 1974 (26) showed that IO_4^- could also act indirectly by modifying the surface of lymphocytes or macrophages and that this alteration would change the surface membrane sufficiently so that lymphocytes from the same donor would be stimulated by these periodate stimulated cells.

(f) Enzymes

i) Neuraminidase and Galactose Oxidase

Novogrodsky and Katchalski showed that the formation of aldehyde groups on the surface of lymphocytes gave rise to blastogenic transformation. They were able to obtain blastogenesis by incubating mouse spleen cells with neuraminidase which exposes the galactosyl residues on the cell surface. The cells were subsequently incubated with galactose oxidase which formed aldehyde groupings from the galactose residues exposed by the neuraminidase (27).

ii) Trypsin

The first report about the possible mitogenic activity of trypsin came from Mazzei and his collaborators (28) who showed that trypsin and chymotrypsin injected *in vivo* caused the appearance of 2% to 14% blasts in cultures of human lymphocytes. Subsequently, in another report, it was shown that papain had a qualitatively similar effect (29). These findings raised no interest among researchers until Hough and Stevenson, in 1973, said that trypsin did not produce any measurable response from human lymphocytes (30). Then Visher (5) and Kaplan and Bona (6) reported that tryptic treatment of mouse spleen lymphocytes caused a blastogenesis. The latter authors reported that treatment of mouse spleen lymphocytes with trypsin at a concentration ranging from 0.1 to 1.0 μg/ml was found to cause significant stimulation in the incorporation of ^{3}H thymidine and even higher amounts of incorporation were noted when trypsin was incubated with nude mouse spleen cells (mice that have a genetic defect and that are born without functional thymus and are also characterized by the fact that they

do not have any hair). Trypsin was without effect on mouse thymic lymphocytes but on the other hand caused small but significant activation of human peripheral blood lymphocytes. At the end of their report, the authors conclude that the trypsin stimulates the B derived lymphocytes.

More recently, Hart and Streilein reported similar observations regarding the action of trypsin on hamster lymphocytes (31). Trypsin was also shown by Lindahl-Kiessling & Peterson (32) to eliminate reversibly the ability of lymphocytes to respond to PHA. Also, Vischer, Bretz and Baggiolini (33) have shown that human polymorphonuclear leukocytes contained large amounts of neutral proteinases. They strongly suggest that the stimulatory effect of these enzymes on human and mouse lymphocytes is due to the proteolytic activity of these enzymes.

2) Antigens

Normally the lymphocytes in peripheral blood undergo blastogenesis only when they meet the proper antigen. It is well known that in order to react, the lymphocyte must be "sensitized" to the particular

determining antigenic site of the infecting agent. Then the response becomes specific and only a certain restricted population of lymphocytes will transform. In vitro, it is possible to recreate these conditions by adding to the cultured lymphocytes certain extracts from microorganisms capable of provoking the synthesis of antibodies or of cell proliferation. Examples of specific antigens used in vitro are: streptolysin O (SLO) and Varidase (VAR).

3) Mixed Lymphocyte Reaction

In 1964, a Canadian researcher, Barbara Bain showed that if lymphocytes of a certain donor were cultured in the presence of lymphocytes coming from another donor, a blast transformation would occur. Normally, this reaction would be "two-way" since each population would be acting as stimulator and responder (34). She also noted that the activation was associated with the transplantation antigens on the surface of the lymphocytes. This general phenomenon was then called the mixed lymphocyte reaction (MLR). The changes occurring in the lymphocytes, stimulated in such a way, are similar to those in the antigen stimulated cultures except that fewer cells respond and that the peak of activity of the stimulated cells is usually between day 5 and 7.

II. ANTI PROTEASE

As a test of Kaplan and Bona's hypothesis (6), I have studied the effect of protease inhibitors on human peripheral blood lymphocyte and on mouse spleen lymphocyte transformation. The protease inhibitors used were both protein and synthetic.

A - Protein Inhibitors

1) Soybean Trypsin Inhibitor (SBI)

The soybean contains several protease inhibitors of which two are now well characterized and studied.

(a) Bowman-Birk SBI

The Bowman-Birk SBI was first found by Ham and Sandstedt in 1944 (35) and a few months later (and what seemed like an independent finding) by Bowman (36). Birk (37) was the first to characterize the inhibitor which consists of 71 amino acids residues including 14 half cysteine residues from which derives the surprising stability to heat, acid and proteolytic digestion of this inhibitor. This inhibitor, with a molecular weight of roughly 8,000, was shown to contain two independent inhibitory sites for trypsin (Lys^{16} -Ser^{17}) and chymotrypsin (Leu^{43} -Ser^{44}). It can be divided in 2 small fragments having either trypsin or chymotrypsin inhibitory activity (38).

Antibody made against Bowman-Birk SBI cross reacts with the trypsin inhibitor extracted from the lima bean but did not show any specificity for the trypsin inhibitor isolated by Kunitz from soybean.

(b) Kunitz SBI

Kunitz SBI isolated by Kunitz (39) contains 181 amino acids residues including two disulfine bridges. The amino acid sequence was determined by Koide and Ikenaka (40,41,42). The inhibitor has a molecular weight of roughly 24,000 and an isolectric point at pH 4.2. Its reactive site is also known to be Arg^{63}--Ile^{64}. The three dimensional structure of the inhibitor may play a great role in its inhibitory ability since Phe can be substituted for the Arg of the active site without affecting the activity of the inhibitor against trypsin.

(c) General Mode of Action

The SBI is a reversible inhibitor. Trypsin is known to hydrolyse the peptide bond between Arg^{63} and Ile^{64} of its reactive site. The structural change occurs in the inhibitor molecule and trypsin becomes reversibly trapped in the enzyme-inhibitor complex. Therefore, the more stable the complex is, the more efficient is the inhibition.

The events of the inhibition can be summarized in the following equations:

$$E + I \underset{k-1}{\overset{k1}{\rightleftharpoons}} C \qquad (1)$$

where E is the enzyme, I the inhibitor and C the enzyme-inhibitor complex. This equation has the following constant of association:

$$K_{ASS} = \frac{[C]}{[E]\ [I]} = \frac{k1}{k-1} \qquad (2)$$

But it was found (43,44) that the enzyme could reversibly interconvert the intact inhibitor into an hydrolysed inhibitor (with the Arg^{63}--Ile^{64} bond hydrolysed) as described by the following equation:

$$C \rightleftharpoons E + Ih \qquad (3)$$

where I_h is the hydrolysed inhibitor. Therefore, the simplest equation describing the mechanism of action would be:

$$E + I \rightleftharpoons C \rightleftharpoons E + Ih \qquad (4)$$

The constant of association of such an equation could then be summarized by the following equation:

$$K_{ASS} = \frac{[C]}{[E]\ ([I] + [Ih])} \qquad (5)$$

The constant of hydrolysis of the inhibitor would then be:

$$Kh = \frac{[Ih]}{[I]} \qquad (6)$$

This equation then predicts that the conversion of I to L_h occurs via a single intermediate and that the formation of C

will be a second order reaction regardless of the reactant concentration. This was shown (45,46) to be true but at small reactant concentration, the reaction becomes of the first order type. Therefore, in order to account for this non-covalent complex enzyme-inhibitor an enzyme-inhibitor (hydrolysed) should be added to equation 4.

The easiest way to describe the mechanism of action would then be the following:

$$E + I \underset{k-1}{\overset{k1}{\rightleftharpoons}} L \underset{k-2}{\overset{k2}{\rightleftharpoons}} C \underset{k-3}{\overset{k3}{\rightleftharpoons}} L_h \underset{k-4}{\overset{k4}{\rightleftharpoons}} E + I_h \qquad (7)$$

where L is the non-covalent complex enzyme-inhibitor.

In this type of inhibition, it is very important to note that the hydrolysis of the reactive site peptide bond is not completed. There is instead an equilibrium between the hydrolysed inhibitor I_h and the intact inhibitor I, meaning that the equilibrium constant K_h is then dependent only on the inhibitor used and not the enzyme. The complete mechanism of action of the inhibitor can be therefore divided in two parts: first, the hydrolysis of the peptide bond with no conformational changes occurring and second, the relaxation of the hydrolysed molecule to its lowest free energy conformation. In most proteins, this relaxation is very large since the hydrolysable peptide bond is located in a critical region of the protein and when it is broken, the protein undergoes more drastic conformational changes. Therefore the K_h of most proteins is a high value indicating complete hydrolysis. In the protease

inhibitor however, the hydrolysable bonds are located in very rigid regions and therefore little relaxation occurs (low K_h values). The value of K_m is extraordinarily low, about 10-12 at neutral pH, indicating the efficiency of the inhibitor.

2) Ovomucoid (OM)

Lineweaver and Murray (47) were the first ones to find the antitryptic factor of chicken egg white. The purified inhibitor, according to a combination of a data on sedimentation, diffusion, viscosity and partial specific volume, has a molecular weight of about 28,000. Kinetic studies indicate that the inhibition of trypsin by OM is of the non-competitive type. It was found by Fraenkel-Conrat (48) that OM retains almost all its activity after most of its amino groups have been blocked by acetyl groups indicating that these amino groups were not involved in the inhibiting action of OM on trypsin. But on the other hand, the same authors have shown that esterification with methanol caused inactivation of the inhibitor which suggest that the carboxyl groups are essential for its anti-tryptic activity. It also appears that the amino groups of the trypsin are essential for the combination with the OM but not with the substrate. The amino acid sequence of the protein is known along with that of the Arg--Ala specific peptide at the reactive site.

The general mechanism of action of the OM is very similar to the SBI just described. One exception however is the active site peptide bond of the OM which is between Arg and Ala. The constant of hydrolysis of this bond shows the same pH dependence as the SBI and therefore the general mechanism could be described by the same equations used for the SBI. The K_I value is of about 2×10^{-10} as reported by Lineweaver and Murray (47). It is noteworthy that the hydrolysed inhibitors SBI and OM combined with trypsin at much lower rate than the native. In both cases, removal of newly formed COOH terminal residues of the hydrolysed inhibitor renders the product inactive. In addition, the resemblance between these two trypsin inhibitors in their content of disulfide bridges, which render the molecule very stable even after the hydrolysis, is of some importance.

3) Lima Bean Inhibitor (LBI)

It was Tauber and his collaborators, in 1949, who isolated for the first time, from lima bean, a crystalline inhibitor that inhibited proteolysis by trypsin and chymotrypsin (49). Then in 1952, Fraenkel-Conrat and his collaborators, characterized this LBI and calculated, using osmotic pressure and ultra-centrifugation analysis, that the molecular weight was about 10,000 (50).

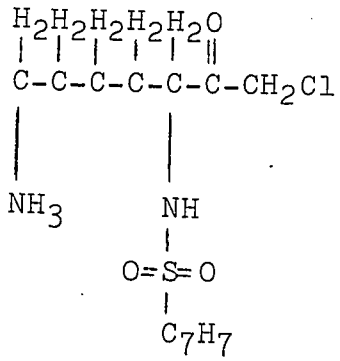
It was then demonstrated by Krahn and Stevens (51) that LBI could form 1:1 molar complex with trypsin and also with chymotrypsin and that the binding site of these enzymes were different and independent. It was subsequently showed by the same authors that the trypsin sensitive bond was Lys^{28}--Ser^{29} (52), while the chymotrypsin sensitive bond was thought to be Leu^{53}--Ser^{54}. But Krahn and Stevens have now brought some new evidence that the chymotrypsin specific peptide bond is probably Phe^{53}--Ser^{54}.

Like the Bowman-Birk soybean inhibitor, the LBI is a double headed inhibitor. But the resemblance does not stop there. Indeed the two inhibitors show some striking points of similarities: first, they have approximately the same molecular weight, second, they both inhibit trypsin and chymotrypsin in the same manner (formation of a complex enzyme-inhibitor, see 11-A-(1)-b), third, the amino acid sequence of both inhibitors is almost the same and finally, antibodies made against LBI intereact with the Bowman-Birk SBI and vice-versa, but not with the other SBI inhibitor (Kunitz).

It seems therefore, that the legume seeds contain very stable and similar protease inhibitors that act as a defence mechanism of legume seeds against insects.

B - Synthetic Inhibitors

1) N-tosyl-L-lysine-chloromethyl Ketone (TLCK)



(TLCK)

TLCK first synthesized by Shaw et al (53) was used as an active center labelling reagent for trypsin. Shaw and his collaborators showed that TLCK modified the active site His^{46} of the trypsin irreversibly and that only one of the three histidines of the enzyme was affected by the inhibitor. The compound inactivates trypsin by a substrate like behavior, forming a complex in which the alkylation of the active center histidine takes place irreversibly. The reaction can be summarized by the following equation:

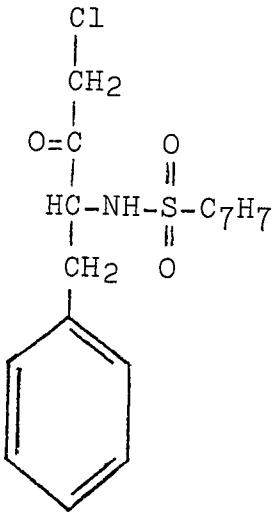
$$I + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} I.E \xrightarrow{k_{-2}} E_{alkylated}$$

Shaw and Glover (54) reported that the inactivation of B-trypsin at pH 7.0, 25° by TLCK, at various concentration of the inhibitor, was observed to obey first-order kinetics. They calculated a K_I of 2.1×10^{-4}

TLCK is considered to be stable in acid pH (Shaw et al) but decomposes very fast in alkaline pH; 48% of TLCK is decomposed in 5 minutes at pH 9.0. Since the pH of the culture medium used varied between 6.8 to 7.2 for all experiments, the stability of the compound will not be questioned.

It is also noteworthy that thrombin, plasmin, and acrosin can be inhibited by TLCK. Chymotrypsin on the other hand is not affected by the compound (53).

2) Tosyl-phenylalanyl-chloromethyl ketone (TPCK)



(TPCK)

As TLCK for trypsin, TPCK was first synthesized to study the active site of chymotrypsin (55). The irreversible inhibitor, also a substrate analogue, acts in a similar fashion to TLCK, i.e. by forming an enzyme inhibitor complex after alkylating the active site histidine of the enzyme. It specifically inhibits chymotrypsin and only the active enzyme (not chymotrypsinogen).

The optimum pH for the inhibitory reaction is pH 7.2 (55), pH at which TPCK seems fairly stable. The trypsin remains unaffected by this agent as demonstrated by Schoellmann and Shaw (55). It was also found that the inhibitor and the enzyme reacted in a one to one ratio.

3) Phenyl Methyl Sulfonyl Fluoride (PMSF)

Studies undertaken to elucidate the mode of action of protease inhibitor PMSF were performed by Fahrney and Gold (56). They were able to extend the work done by Hartley and his collaborators who showed that dimethylamino-naphthalene-5-sulfonyl chloride can sulfonate the active site of chymotrypsin and proceeded to show that if sulfonyl fluoride is in large excess of the enzyme concentration, the rate of inactivation of the enzyme would be proportional to the concentration of the sulfonyl fluoride. Fahrney and Gold also give evidence that the inhibition proceeds by a specific pathway where two important factors determine the effectiveness of PMSF as a protease inhibitor: first, the affinity of the compound once bound at the site can sulfonate it.

$$\underset{E}{ENZ} + \underset{I}{RSO_2F} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{EI}{ENZ\text{-----}RSO_2F} \xrightarrow{k_2} ENZ + \underset{P}{SOR} + F^-$$

En résumé, PMSF causes inhibition of proteases especially trypsin and chymotrypsin by causing the sulfonation of the reactive groups of serine. PMSF can also react with the serine residues outside the active site.

MATERIALS AND METHODS

I. CELL CULTURE

Human peripheral blood lymphocytes were the most studied cells in this work, although the reactions of mouse or rat cells were also investigated.

Human blood was usually taken in the morning and the leukocytes cultured in the following few hours. The blood was withdrawn, with an heparinized syringe, from the peripheral veins of normal volunteers.

Mice bred by the Faculty of Medicine of the University of Ottawa, and coming from the mouse line Paris R-3 (7 years inbred) also served as donors. After the mouse was killed (by decerebration) the cells were obtained by aseptically removing the spleen from which splenocytes were withdrawn.

A. Human Cells

1) Purification methods

a) Dextran

The freshly drawn blood was distributed into glass vials containing enough dextran to make a 25% solution after the addition of the blood. These vials were sedimented at unit gravity at 37° for 45 minutes. This incubation separated the plasma and the red cells. The leukocyte rich plasma was then removed and centrifuged. The cells were resuspended in a small volume of

the same medium used in the experiment. An aliquot of this cell suspension was diluted in a solution of 2% acetic acid and 0.01% gentian violet in water, which stained the nucleus of the cells. The mononuclear cells were then counted. Using this method, usually 20% to 25% (depending on the donor) of the total cell counts were mononuclear cells. The cell cultures were then made according to the total mononuclear cell count. Cell viability was done using trypan blue exclusion test.

b) Ficoll Hypaque

The ficoll hypaque purification method was used whenever relatively pure population of lymphocytes were needed. In this particular method, the freshly drawn blood was diluted in an equivalent volume of culture medium. Twenty-five (25) ml. of this blood solution was then carefully layered on 15 ml. of Ficoll Paque (Pharmacia) contained in a 40 ml. round bottom Sorvall centrifuge tube. The layered blood was then centrifuged using a Sorvall RC-2B at 400_g for 35 minutes and at room temperature. This centrifugation caused the blood components to separate in the following manner: the red cells formed a large pellet at the bottom of the tube, the granulocytes sediment on top of the red cells, and a white band consisting of lymphocytes is clearly seen in the plasma-Ficoll Paque solution on top of it. The plasma-Ficoll Paque solution on top of the tube was removed using a pasteur pipette and the white bands

were pooled in one tube to which the medium was added (3 times the amount of lymphocyte solution). The diluted lymphocyte solution was then centrifuged at 500g for 10 minutes in the same conditions as described earlier. This centrifugation resulted in a pellet formed by the lymphocytes at the bottom of the tube. The supernatant was discarded and the lymphocytes were dispersed in a small quantity of culture medium.

They were then counted and made up to a concentration of 1×10^6 per ml. This method yielded an almost pure lymphocyte suspension (96%-97% pure) and proved to be much more efficient than the dextran purification method. Although the Ficoll Hypaque purification method was most commonly used in this work, a second method of lymphocytes purification was also utilized. This method consisted in the passage of leukocyte rich plasma through a glass syringe partly packed with glass wool. This method had been previously developed by a member of our laboratory (M. Quastel (57)). The cells recovered from this column after 30 minutes incubation appeared to be 95% pure lymphocytes. These cells when cultured showed normal response to mitogens and sometimes, depending on the experiment, showed some response to antigen stimulation.

c) Purification of Macrophages

This method of purifying macrophages was first devised by Alter and Bach (18), but was used as described by Wright (58). It consists in incubating unpurified leukocytes in medium 199 plus 30% autologous plasma in a plastic petri dish and incubating in a humidified plastic box containing 5% CO_2 for 4 to 6 hours. Then PBS plus 10% plasma was used twice in washing the non adherent cells. The plates were then incubated for another 24 hours after which the plates were washed with PBS in order to remove, with the aid of a rubber policeman, adherent cells. Contrary to the method of Alter and Bach, no trypsin was used to remove the adherent cells. These cells were then washed and resuspended in medium containing heparin (to prevent clumping) and counted. These "purified" macrophages were then added back to the lymphocyte culture in a ratio varying from 1/10 to 1/100 depending on the experiment.

2) Preparation of Cell Culture

a) Culture Conditions

For most of the experiments, lymphocytes were cultured in microplates. The cultures were made up (unless otherwise specified) in medium with 15% autologous plasma. Aliquots of 200 µl were distributed using an Oxford pipetter, to the wells of the microplate, filling about 2/3 of the well.

The plates were then carefully covered with a plastic lid. For the first 50 experiments or so, the plates were kept in a small plastic box with a gas phase of humidified 5% CO_2 in air, in an ordinary incubator until harvested. Thereafter, since the laboratory had purchased a CO_2 incubator (Hot Pack) the plates were then kept in this incubator without the presence of these plastic boxes. (see Table I for sources of materials).

b) Antibiotics and Prevention of Contamination

All experiments were carried out in a laminar flow hood (Conn. Environmental R & D Corp., New Britain, Conn.) to reduce the risk of contamination of the cultures. Even though the manipulations were done with sterile precautions, the antibiotics penicillin and streptomycin were sometimes added to the culture medium.

c) Medium

TC 199 was usually used in this work. It was essentially used for mitogen stimulation experiments while RPMI 1640 was used for antigen stimulation and MLR.

3) Mitogens and Antigens

Mitogens and antigens were added to the culture medium before its distribution to the wells. The standard doses were selected on the basis of the response

TABLE 1

Sources of Materials

Name	Number	Source	
Heparin	50770	General Biochemicals	Chargin Falls, Ohio
Dextran (Macrodex, 6% in saline)		Pharmacia	Montréal, P.Q.
TC 199	115E	Gibco	Grand Island, N.Y.
	14-18017	Connaught	Willowdale, Ontario
RPMI 1640	1876	Gibco	Grand Island, N.Y.
	1487-01-7	Connaught	Willowdale, Ontario
FCS	614HI	Gibco	Grand Island, N.Y.
	2601-02-7	Connaught	Willowdale, Ontario
Culture tubes		Falcon Plastics	Oxnard, California
Microplates & lids	3040,3041	"	"
M.A.S.H.		Otto Hiller	Madison, Wisconsin
Phycoll Paque	784021	Pharmacia	Montréal, P.Q.
Penicillin-Streptomycin		Difco	Detroit, Michigan
LCS vials		New England Nuclear	Dorval, P.Q.
Econofluor	NEF 941	"	"
PHA-M	05-2857	Difco	Detroit, Michigan
LAG	736017	Pharmacia	Montréal, P.Q.
PWM	536	Gibco	Grand Island, N.Y.
Con A	234567	Calbiochem	LaJolla, California
LPS	3126-25	Difco	Detroit, Michigan
10^{-4} Var (SKSD)		Analar	
SLO (Streptolysin-O reagent)		Lederle	Pearl River, N.Y.
		Gibco	Grand Island, N.Y.
Protease Inhibitors (see Table 3)			
CNBr activated Sepharose 4B		Pharmacia	Montréal, P.Q.
Trypan blue	525	Gibco	Grand Island, N.Y.
Filters (for M.A.S.H.)	9-874-32	Whatman	

experiments done by P. Wright while working in our laboratory. Table 2 gives a list of the standard mitogens and their doses used, given as ml of stock made up according to the manufacturer instructions, per ml of culture.

TABLE 2

Standard Doses of Mitogens and Antigens

Mitogen	ml stock/ml culture volume used
PHA	0.02
LAG	0.025
ConA	0.025
PWM	0.02
LPS	0.025
SLO	0.02
VAR	0.02

B. Mouse Cells

1) Purification of Leukocytes

The mice used in these investigations were killed by decerebration and their spleens were removed aseptically. The mesentery was cut free from the spleen which was washed in a sterile solution of isotonic NaCl (0.17M). The spleen was then transferred to a second dish, containing PBS and 10% fetal calf serum (FCS), where it was teased and dispersed with the aid of tweezers. The resulting solution (debris and splenocytes)

was then layered on top of 4 ml of 100% FCS for 10 minutes. During this period, all debris (coming from the ruptured spleen) settled in the bottom of the tube. The resulting layer (remaining on top of the tube) was then transferred to another tube containing 100% FCS and centrifuged for 7 minutes at 400 g. The resulting pellet was then dispersed in a solution ammonium chloride (to destroy the red cells). After recentrifugation the cells were counted in exactly the same manner as the human cells.

2) Preparation of Cell Cultures

The medium used when dealing with mouse cells was always RPMI 1640 (Gibco and Connaught) supplemented with 10% FCS. Antibiotics (penicillin and streptomycin; Difco) were always added to the culture medium. The cells were cultured in microplates in the same way as the human cells, but at a concentration of 2×10^6 per ml. In general the mouse cells were exposed to the same conditions as the human cells. (Same incubator, medium, antibiotics, harvesting techniques, etc.).

II. HARVESTING

Cultures of human lymphocytes stimulated by mitogens were usually harvested at day 3, while antigen stimulated cells and cells involved in MLR were harvested from day 5 to 7 depending on the donor. Mouse cells were usually harvested from day 2 to 3. The harvesting of the microplate culture (12 wells at a time) was done using a multiple automatic simple harvester (MASH) designed for that purpose (59). After brief washes with distilled water the glass fiber strips (Whatman GF/C) were removed and left to dry. The filters were then submerged in a solution of Econofluor (toluene based pre-mixed cocktail; NEN) contained in minivials (Fisher).

Counting was done on a Beckman LS233 scintillation counter at ambiant temperature. Usually samples were automatically counted for 2 mins. or 1% efficiency whichever came first, but in some experiences where the number of counts per minute was low, each sample was counted for 5 mins.

III. PREPARATION OF THE INHIBITOR

A. Protein Inhibitor

For each experiment, a fresh solution of protein inhibitor was prepared a few hours before it was added to the culture. Depending on the final concentration needed, the inhibitors were prepared in 2 different stock solutions. For large ($\geq$ 0.5 mg/ml) concentration, the stock solutions were

made up to 10 times the final concentration and therefore 0.1 ml was added to 0.9 ml of culture medium. For smaller concentrations (< 0.5 mg/ml), the stock solution was made up to 40 times the final concentration and 25 μl of inhibitor was then added to 1 ml of culture medium.

All inhibitors were dissolved in the culture medium used in the particular experiment. During the experiment, the inhibitors were kept in the lamillar flow hood and at room temperature.

B. Synthetic Inhibitors

Synthetic Inhibitors, when purchased in powder form, were always prepared just before the experiments. When the inhibitors were obtained in a liquid form, storage was at 4o.

Before each experiment, a stock solution of 40 times the highest concentration was made up in culture medium. 25 μl of the inhibitor solution was then used per ml of culture. (See Table 3 for list of inhibitors).

TABLE 3

Sources of Protease Inhibitors

Name	Number	Source	
SBI	T-9003	Sigma	St. Louis, Misou.
	65035	Calbiochem	LaJolla, Calif.
SBI (crude)	T-9128	Sigma	St. Louis, Misou.
Ovomucoid	T-9253	"	"
Lima Bean Inhibitor		"	"
TLCK	616382	Calbiochem	LaJolla, Calif.
TPCK	616387	"	"
TAME	61628	"	"
PMSF	P-7626	Sigma	St. Louis, Misou.
E Amino Caproic a.	A-2504	"	"
Control Protein (Ovalbumin)	A-2512	"	"

IV. INSOLUBLE SOYBEAN INHIBITOR

A. Preparation

SBI was coupled covalently to sepharose beads using the method developped by Freinstein (60) for ovomucoid. 1.3 g of cyanogen bromide activated sepharose 4B (Pharmacia batch #9787) was swollen and washed for 20 minutes in 10^{-3} M HCl. The beads were then deposited on a glass filter and washed with 250 ml of 0.1 M $NaHCO_3$ buffer pH 8.5, containing

0.5 M NaCl. 24 mg of SBI in 12 ml of the same buffer was then mixed with the gels for 18 hours at 4°. An aliquot of the supernatant was then taken before and after the 18 hours of mixing and the optical density was measured. (Comparison between the supernatant taken before the 18 hours mixing, with the supernatant taken after permitted measurement of the amount of protein that did not bind to the beads). The unbound SBI and the coupling buffer were washed away and the remaining active groups were allowed to react with 20 ml of 1 M ethanolamine at pH 8.0 for 2 hours. After the removal of ethanolamine, the beads were washed 3 times with acetate buffer (0.1 M pH4 containing 1 M NaCl) followed by a borate buffer (0.1 M, pH 8.0, containing 1 M NaCl). Finally, the beads were kept in about 10 ml of coupling buffer at 4°.

Control beads were prepared in the same manner but without the addition of the SBI.

B. Anti-trypsin Activity of the Bound SBI

The antitryptic activity of the insoluble inhibitor was measured by its ability to inhibit the action of trypsin on casein as described by the method of Bergmeyer (61).

V. ANALYSIS OF PURITY

To verify the purity of the different commercial preparations of SBI, various methods were used.

A. Gel Electrophoresis

1) SDS gel electrophoresis

The method used to run the SDS gel electrophoresis is that of Fairbanks (62). The different chemicals and the composition of the gels and of the several buffers used are described in Tables 4 and 5.

TABLE 4

List of Materials for Purification Procedures

Acrylamide	Eastman	
Bis	"	
Bromophenol blue	Nutritional Biochemical Corporation	
Coomassie Brilliant Blue	Bio Rad	
DTT		
SDS	Sigma	St. Louis, Misou.
Sulfacylic acid	Fisher	Ottawa, Ontario
TEMED	Eastman	
Tris	Backer	

TABLE 5

Composition of SDS Gels and Buffers

Stock solution

Acrylamide:

acrylamide 40 g.
Bis 1.5 g.
H_2O to 100 ml.

Buffer X 10:

1 M Tris (40 ml.)
2 M Sodium Acetate (10 ml.)
.2 M EDTA (10 ml.)
Acetic acid to pH 7.4
H_2O to 100 ml.

Running buffer

Buffer X 10 (100 ml.)
20% SDS (1 ml.)
H_2O (850 ml.)

Gels (10 ml. of 5.6% acrylamide solution)

acrylamide 1.4 ml.
buffer X 10 1.0 ml.
20% SDS 0.5 ml.
1.5% Ammonium Sulfate 1.0 ml.
0.5% TEMED 0.5 ml.
H_2O 5.6 ml.

Covering buffer

0.1% SDS
0.15% ammonium persulfate
0.05% TEMED

Sampling buffer

1.0% SDS
5-10% sucrose
10 mM Tris HCl (pH 8)
1 mM EDTA
40 mM DTT
10 ug/ml. Pyronin Y

After polymerization of the gels was complete, 50 ul of the processed SBI sample (prepared by adding 50 ul of a 2 mg/ml solution of SBI to 150 ul of sampling buffer and then boiling for 5 minutes) was layered on the gels covered by the running buffer. 7-8 mA per gel were then applied for about 2 hours. As soon as the marker (pyronin Y) arrived at the bottom of the gel, the current was stopped. The gels were taken out of the glass tube and the bottom of the marker band was marded with china ink.

The gels were then placed in a solution of 25% isopropyl alcohol, 10% acetic acid and 0.05% Coomasie blue overnight at room temperature. After that, the gels were transferred to a solution of 10% isopropyl alcohol, 10% acetic acid and 0.005% Coomasie blue for 6 to 9 hours after which they were transferred again, this time to a solution of 10% acetic acid and 0.0025% or less Coomasie blue. They were left overnight to be finally transferred to a solution of 10% acetic acid until the gels became clear.

B. Normal Gel Electrophoresis

The method of Davis (63) was mostly followed. The chemicals were the same as the ones used in the SDS gels except for the marker Pyronin Y which was replaced by bromophenol blue. Composition of the gels and buffers is shown in Table 6.

TABLE 6

Composition of Normal Gels and Buffers

Stock solution	
Acrylamide:	acrylamide 28.0 g. Bis 0.735 g. H_2O to 100 ml.
Solution A:	1 N HCL (48 ml.) Tris (36.6 g.) TEMED 0.23 ml.) H_2O to 100 ml. pH 8.9
Running buffer	
	Tris 6 g. Glycine 28.8 g. H_2O to 1 liter pH 8.3
Gels (12 ml.)	
	2 ml. of solution A 4 ml. acrylamide 8 ml. ammonium sulfate 8 ml. H_2O
Covering buffer	
	H_2O
Sampling buffer	
	5-10% sucrose in H_2O
Marker	
	Very pale blue solution of bromophenol blue in running buffer.

The gels were, in general, run in the same way as the SDS gels except for a few differences: first, the samples were not boiled before being applied to the gels, second, the marker bromophenol blue was added to the running buffer, third, 6 mA per gels were applied, and finally, when the marker arrived at the bottom of the gels, the gels were taken out and put in 12.5% TCA for 20 minutes.

C. Column Chromatography

The purity of the SBI was also verified using an anion exchange cellulose (Bio Rad Cellex D 0.70 meq/g) column. The cellulose was first washed with 0.5 M HCl and 0.5 M KOH then neutralized and washed several times in a 10 mM potassium phosphate buffer of pH 7.6. The column used had a diameter of 1 cm and height of 15 cm.

A stepwise elution was performed at room temperature. The samples were dissolved in 0.01 M phosphate buffer at pH 7.6 which also served as the starting buffer. After the samples had been deposited on the column and the starting buffer run through, appropriate volumes of 0.034 M, 0.13 M, 0.17 M, and 0.25 M NaCl were passed through the column sequentially.

The column was attached to a LKB fraction collector equipped with a UV monitor. Fractions of 3 ml were taken. The optical density of every fraction was then measured at 280 nm.

D. Ultracentrifugation Analysis

A Beckman Model E ultracentrifuge equipped with the Schlieren optical system was used in these studies. In a first run, the inhibitor was dissolved in water at a concentration of 5 mg per ml. The sample was then centrifuged on a AN-D Beckman rotor at 56,000 rpm at room temperature for 2 hours. The bar angle used was 50° and one picture was taken every 4 minutes.

In another run, the inhibitor (5 mg/ml) was first boiled for 5 mins. in a solution of 1% SDS, 1 mM EDTA, 10 mM Tris HCl, 40 mM DTT, before being centrifuged. In this case the bar angle was 65° and a picture was taken every 16 minutes. The rest of the conditions were identical to the first run.

VI. IMMUNODIFFUSION

Ordinary glass microscopic slides were first coated with a thin layer of .2% agar. After the first layer had dried, the slides were covered with a second layer of agar (this time 2%) containing .01% sodium azide. Eight wells were then made in the agar and distributed symmetrically as seen in Figure 1.

The center wells were filled with rabbit anti-bovine trypsin antibodies (Cappel Laboratories Inc.) while the other wells were filled with the different samples to be tested.

The plates were left at room temperature and in a plastic box where a humid atmosphere was provided. After 24 to 48 hours, the plates were taken out and checked for any signs of precipitation (white bands between the wells).

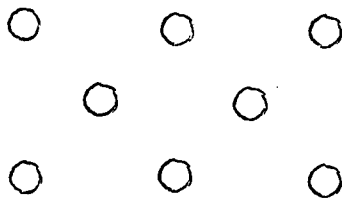


Fig 1: Distribution of the wells in an antigen-antibody diffusion test.

VII. USE OF RADIOACTIVE TRACERS

In all experiments, lymphocyte stimulation was measured by the incorporation of radioactive nucleic acid precursors. In most of the experiments ^{3}H-thymidine which is presumably incorporated into the DNA of the cells was used. The specific activity of the ^{3}H-thymidine is described in in Table 7. Usually 50ul of the thymidine solution was added to every well of the plate 4 hours before harvesting.

In some experiments, ^{3}H uridine (specific activity described in Table 7) was used to measure lymphocyte stimulation. Here too, 50 ul of the radioactive uridine solution was added to each well. Originally, ^{3}H uridine was added to the cultures 18 hours before harvesting, but when uridine with a greater specific activity (see Table 7) was purchased, the incorporation was cut down to 4 hours.

All isotope solutions were made up in culture media and were added using sterile techniques. During incorporation period, the cultures were incubated at 37°.

Additionally, in a few experiments, radioactive amino acids were used.

They were utilized in the same way as the nucleic acid precursors.

TABLE 7

Standard Doses of Radioactive Precursors

Precursors	Catalogue Number	Specific Activity	Concentration in culture
Thymidine-methyl-^{3}H	NET 027	6.7 Ci/mmole	1 µCi/ml
Thymidine-methyl-^{3}H	NET 027X	20 Ci/mmole	1 µCi/ml
Uridine-^{3}H	NET 028	5-15Ci/mmole	1 µCi/ml
Uridine-5-^{3}H	NET 174	25 Ci/mmole	1 µCi/ml.

All radioactive precursors were bought from New England Nuclear, Dorval, Québec.

VIII. TREATMENT OF DATA

There were generally 4 replicate samples in each microplate. These replicates were always set up in the same way from experiment to experiment. After samples had been counted and corrected for background, the mean counts of four replicates were compared to the mean of the appropriate control replicates. The standard deviation was always calculated. When the difference between the two sets of quadruplets was small, the student "T" test was performed at a confidence level of 95%.

The results of the different samples were usually expressed as counts per minutes (cpm) and compared to the cpm of the control samples. Sometimes, the amount of stimulation was expressed as the stimulation index. This was calculated by dividing the mean counts of a quadruplet by the mean count of the control quadruplet.

R E S U L T S

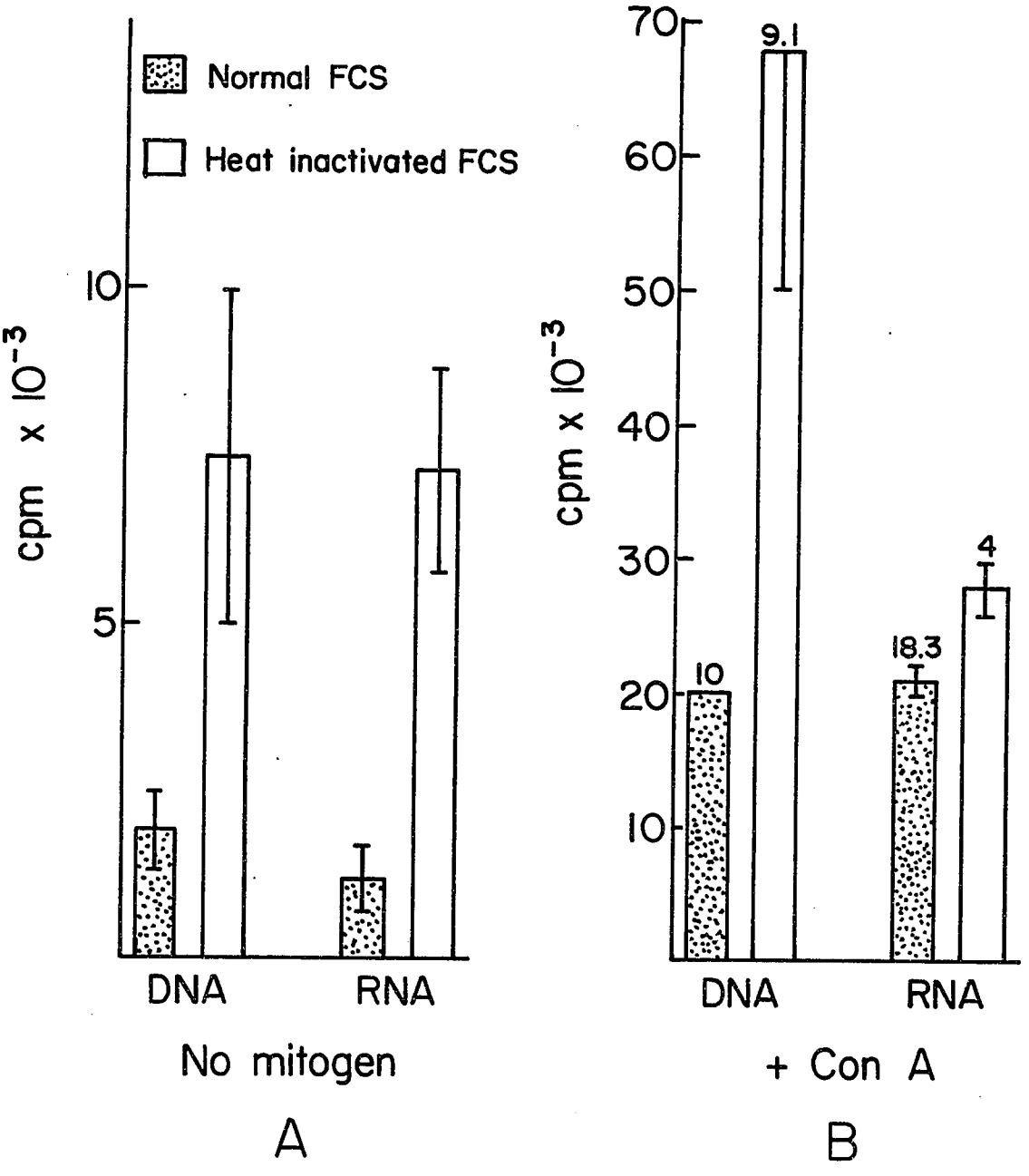
I. CHARACTERIZATION OF HUMAN CELL CULTURE

The characterization of the optimal conditions of human lymphocyte culture in our laboratory had already been done by my colleague P. Wright (58). Therefore, the cell concentration, mitogen concentration, concentration of plasma included in the culture, type of medium, choice of the culture vessel, harvesting techniques, concentration of radioisotopes and the time of harvesting were selected on the basis of her previous experience. The techniques used were comparable to those used in other laboratories and therefore made possible, to a certain extent, the comparison of data. In the work dealing with mice, the conditions were defined mostly by L'Anglais and Bard working in our laboratory and, to a certain extent, by myself. (Conditions are described in Materials and Methods).

Since it was impossible to culture mouse cells with autologous plasma, fetal calf serum (FCS) was used. The two types of FCS available, normal and heat inactivated were tried. Since the two cultures treated with different serum had about the same stimulation index (cpm of stimulated cells/cpm of resting cells) normal FCS was then used in subsequent experiments because it permitted lower incorporation of thymidine and uridine in unstimulated control cultures as shown in Fig. 2.1, Part a.

Figure 2.1: Effect of fetal calf serum on mouse cell cultures.

^{3}H-thymidine and ^{3}H-uridine incorporation into mouse lymphocyte cultures. Part A shows the effect of FCS on unstimulated cells. Part B shows the effect of FCS on ConA stimulated cultures. The stimulation index (see section Materials and Methods, VIII) is shown on top of every column. Cells were harvested at 72 hrs. of culture.


Normal FCS
Heat inactivated FCS
cpm x 10^{-3}
10
5
DNA
RNA
No mitogen
A
70
60
50
40
30
20
10
9.1
10
18.3
4
DNA
RNA
+ Con A
B

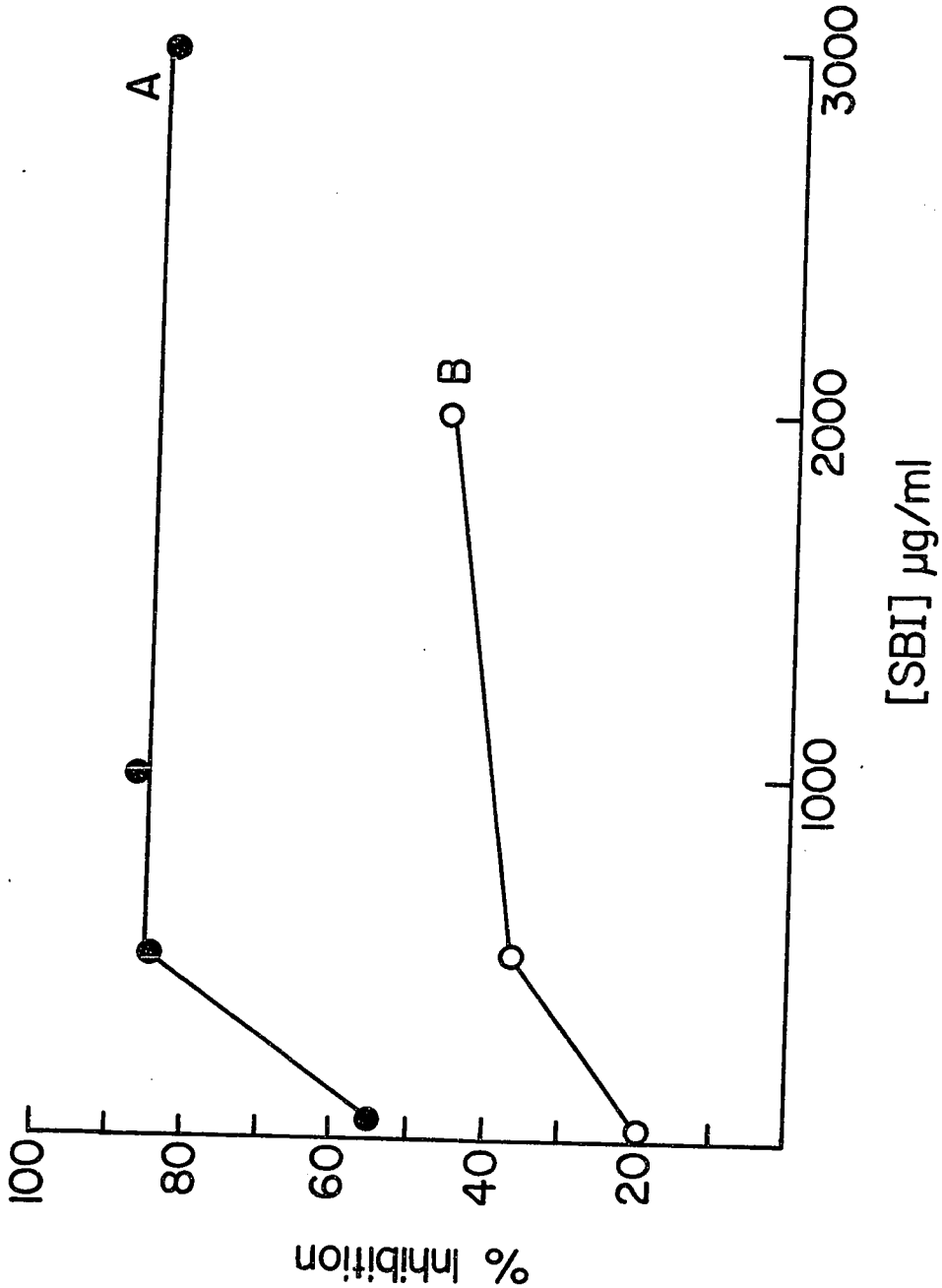
II. VARIABILITY

All the characteristics of the cell cultures (cell concentration, concentration of mitogens, etc.) play a very important role, being the main source of variability of the results in this type of work. Therefore, the conditions were kept constant for almost all experiments but since this work deals with a pool of human subjects, there will always be some variability in the results and this has now been accepted as "normal". Figure 2.2 shows what is meant by variability. These experiments were done using the same donor and exactly the same conditions and show the variability in the action of SBI on lymphycyte blast transformation. In this example, there is no doubt that SBI causes an inhibition of the transformation. The degree of inhibition itself is less important here than the fact that there was a statistically significant difference in the counts of the controls and of the SBI treated cells.

It is very hard to report in a quantitative way a mostly qualitative type of work and the appropriate warning in analysing these results should thus be made. It is noteworthy that for the sake of clarity only one experiment considered to have typical results is usually shown. This method also permitted the expression of the results as cpm and standard deviation of each point to be shown. Whenever more than one experiment is shown on the

Figure 2.2: Variability in SBI inhibition of human lymphocyte stimulation.

Percentage of inhibition of LAG stimulation (measured by ^{3}H-thymidine incorporation) caused by SBI. In experiment A (—●—●—) and B (—○—○—), mitogen and SBI were added on day 0 and the cultures were harvested on day 3.


% Inhibition
100
80
60
40
20
1000
2000
3000
[SBI] µg/ml
A
B

same graph, the results were expressed as percentage of inhibition in order to permit comparison between the different experiments.

III. EFFECTS OF SBI ON STIMULATION OF HUMAN PERIPHERAL LYMPHOCYTES

Kaplan and Bona's hypothesis implies that a proteolytic step was essential to all lymphocyte blast transformation induced by any agent. SBI was thus added to cultures of human lymphocytes stimulated by different mitogens. The protocol of these experiments was very simple: one ml aliquots coming from the stimulated cell culture were distributed in different vials to which different concentration of inhibitor were then added. The cells were subsequently distributed in the culture plates. Figure 3.1 shows that PHA response is inhibited by SBI at concentration ranging from 250 ug/ml to 2000 ug/ml, the latter concentration causing up to 70% inhibition. The effect of SBI on LAG response is shown in Figure 3.2 while Figure 3.3 shows the effect of SBI on the Con A response. SBI also inhibits the response to chemical mitogens (10^{-4}) (see Figure 3.4). Figure 3.5 shows the effect of SBI on the MLR where 2 mg/ml of SBI caused almost 100% inhibition of the thymidine incorporation by the stimulated cells. The response to antigens also seems to be inhibited by the SBI in roughly the same way as for

Figure 3.1: Effect of SBI on PHA stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with SBI and mitogen at day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no SBI). Vertical bars represent the standard deviation.

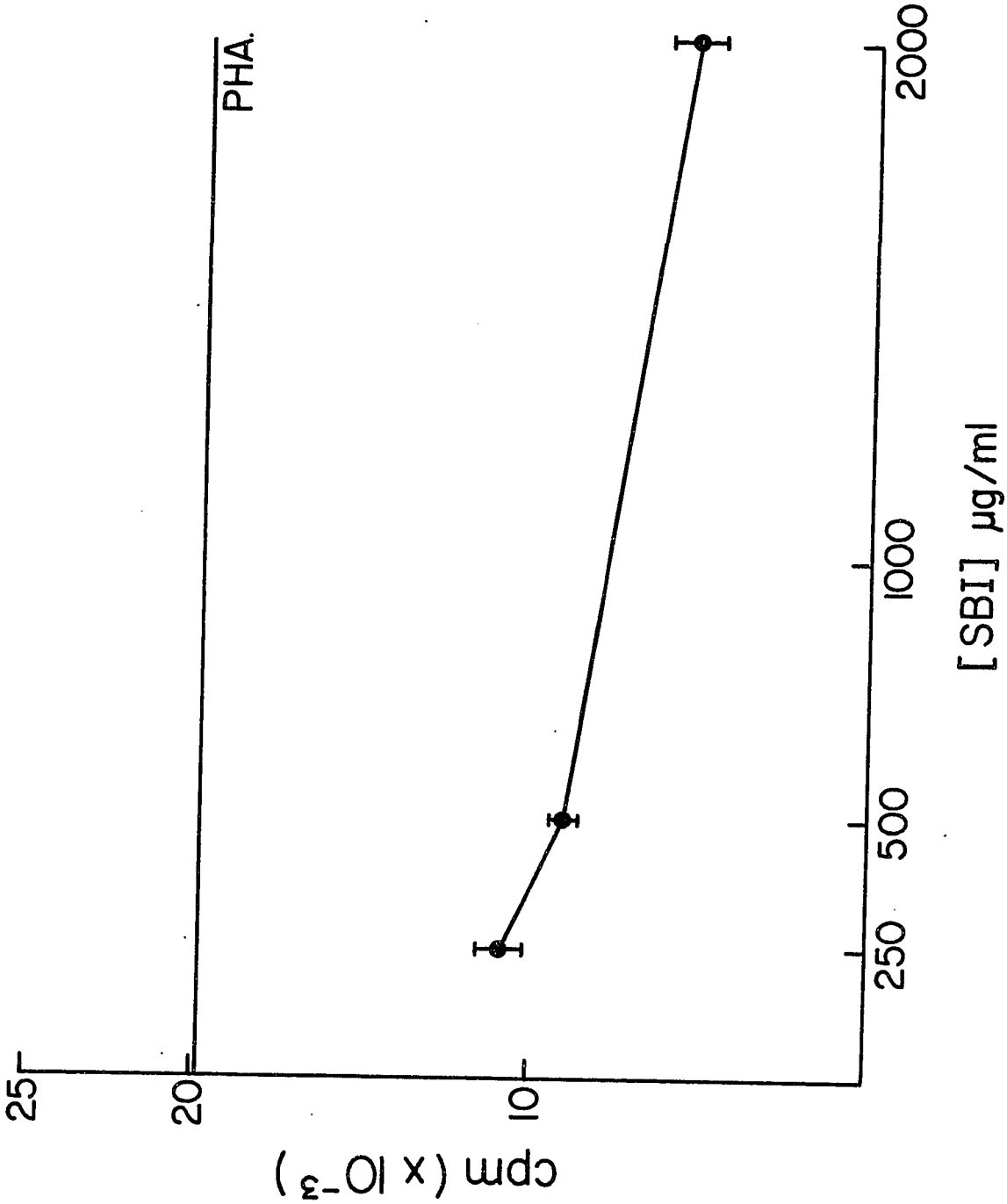

PHA.
25
20
10
cpm (x 10⁻³)
250
500
1000
2000
[SBI] µg/ml

Figure 3.2: Effect of SBI on LAG stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with SBI and mitogen at day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no SBI).

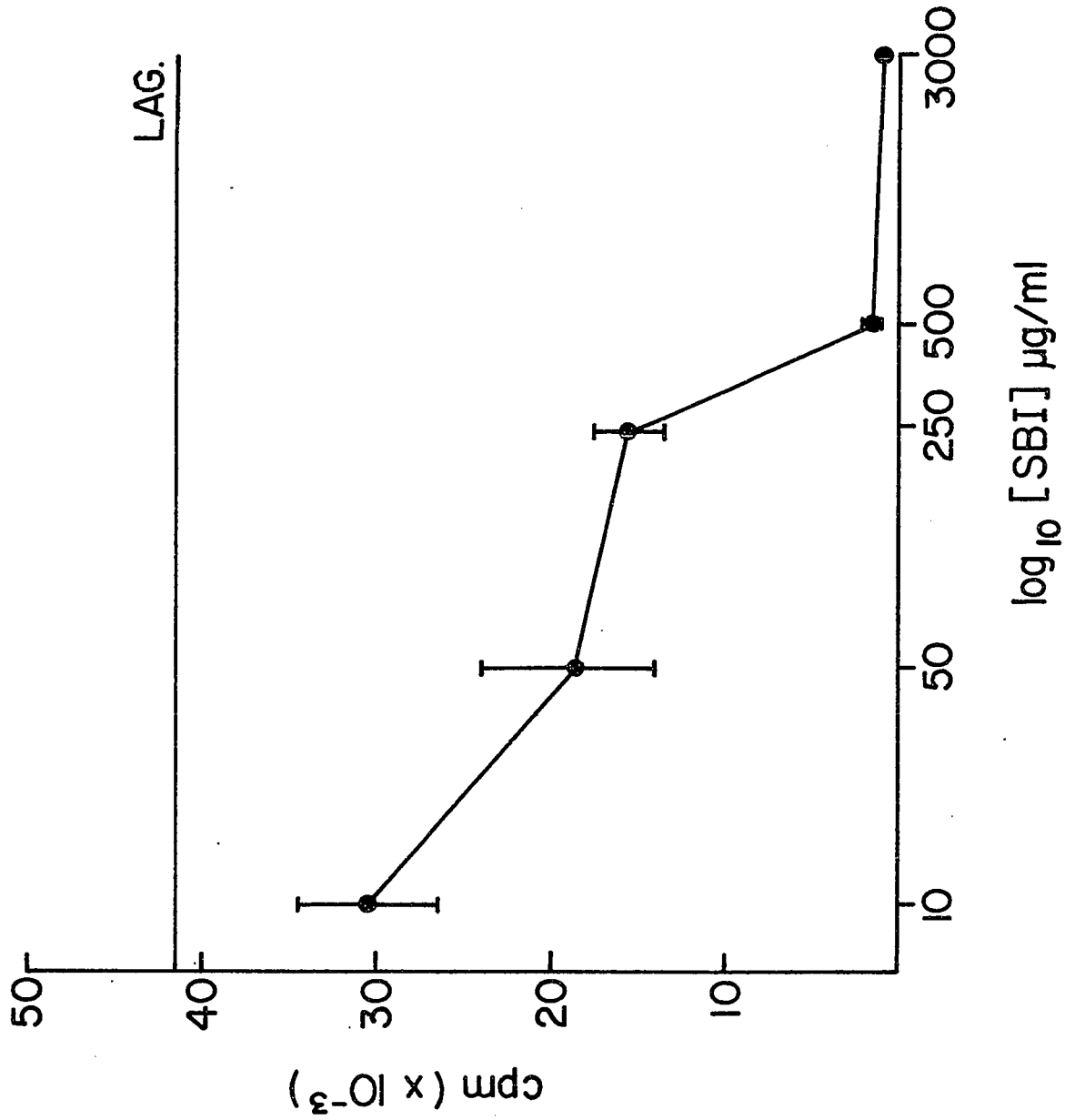

LAG.
cpm (x 10⁻³)
50
40
30
20
10
10
50
250
500
3000
log₁₀ [SBI] µg/ml

Figure 3.3: Effect of SBI on ConA stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with SBI and mitogen at day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no SBI).

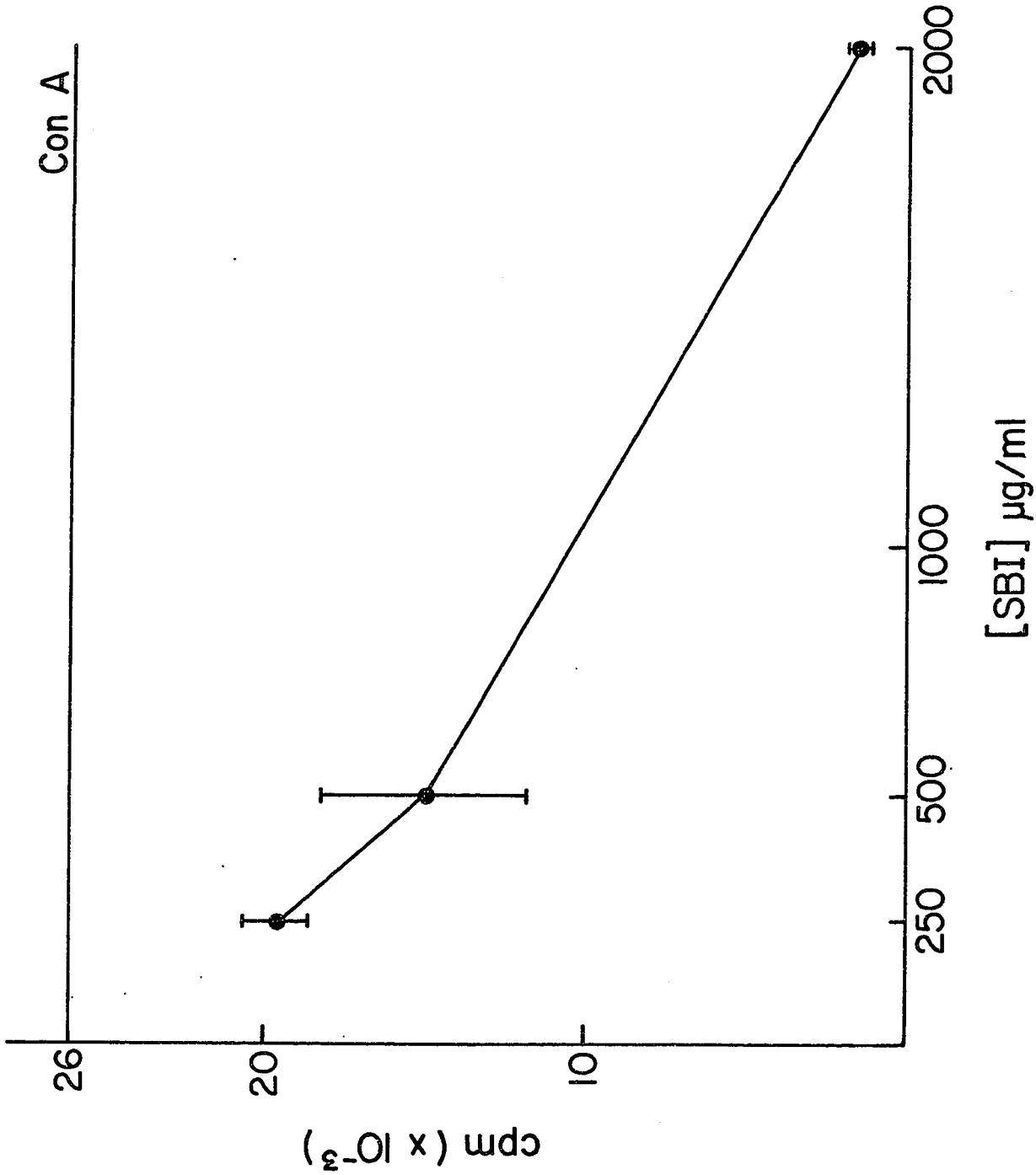

Con A
26
20
10
cpm (x 10⁻³)
250
500
1000
2000
[SBI] µg/ml

Figure 3.4: Effect of SBI on IO_4^- stimulation of human lymphocytes.

^3H-thymidine incorporation in cultures treated with SBI and mitogen at day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no SBI).

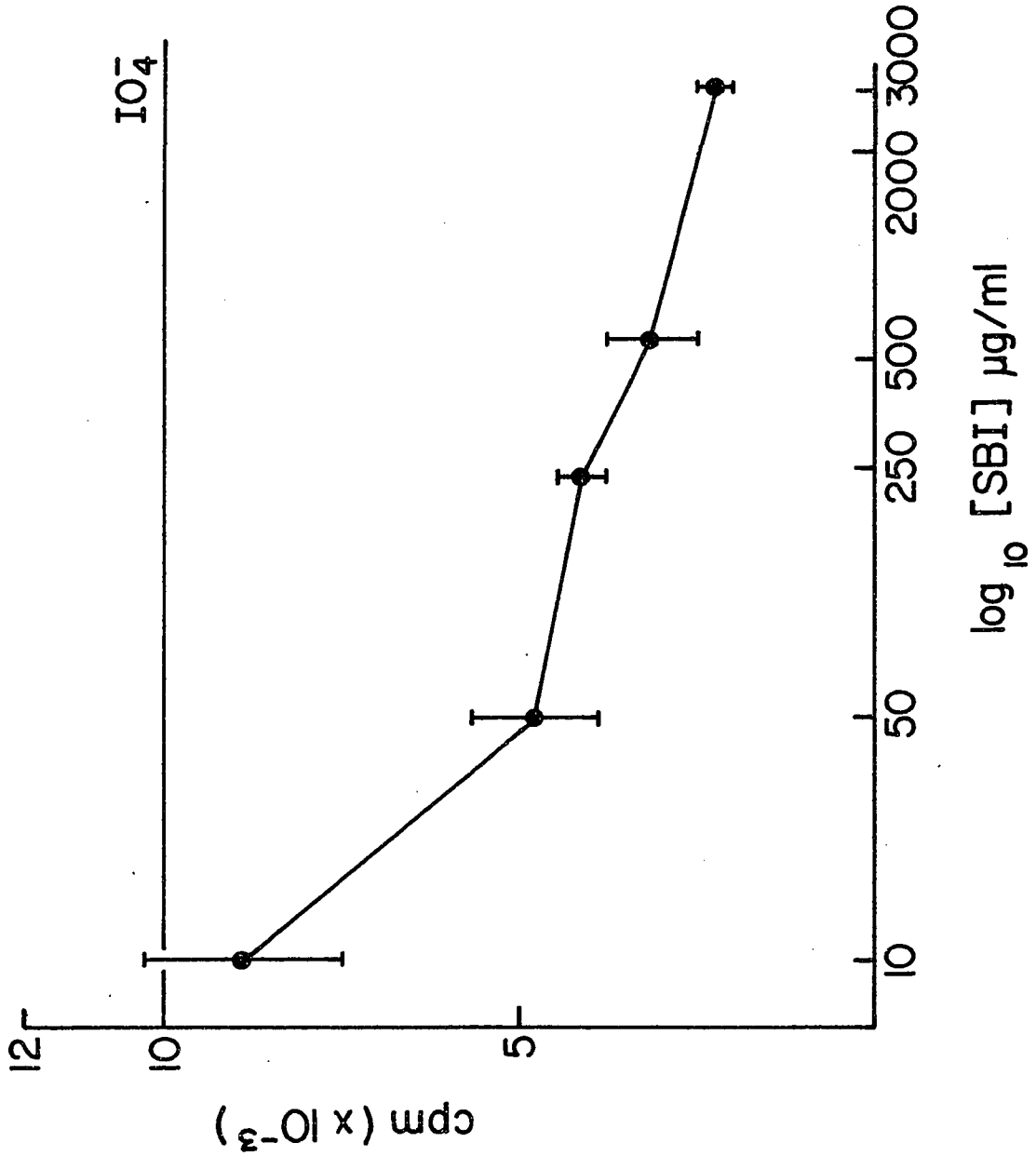
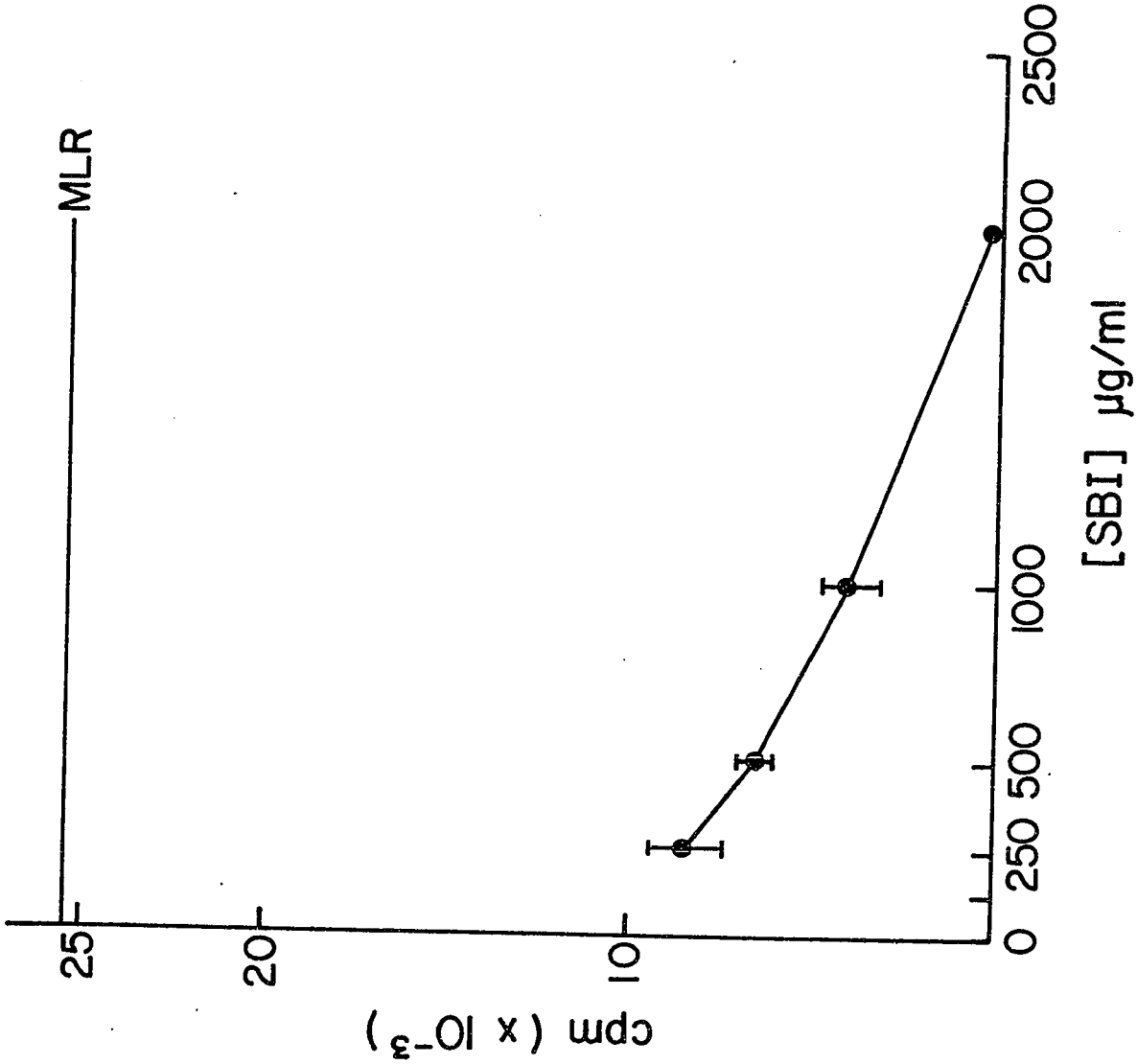

IO_4^-
cpm (x 10^{-3})
12
10
5
10
50
250
500
2000
3000
$\log_{10}$ [SBI] µg/ml

Figure 3.5: Effect of SBI on the mixed (human) lymphocytes reaction.

^{3}H-thymidine incorporation in cultures treated with SBI and mixed at day 0, harvested at day 5. The upper horizontal line represents the average incorporation in stimulated controls.


MLR
25
20
10
cpm (x 10⁻³)
0
250
500
1000
2000
2500
[SBI] µg/ml

the PHA but because of the great variability in the antigen response, it was very hard to draw firm conclusions regarding the effect of the SBI in this particular case.

Thus, it seems that response as measured by the thymidine incorporation in the stimulated cells to mitogens, chemical mitogens, MLR and to a certain extent to the antigen was sensitive to the SBI as predicted by Kaplan and Bona's hypothesis.

IV. ARTIFACTS?

To verify if the inhibition of the response to different stimulating agents was due to the anti-trypsin activity of the SBI preparation or to another non-specific action of the SBI, several tests were done.

A. Viability

Viability studies were done by measuring the number of cells unable to exclude the dye Trypan Blue. In most experiments, the number of cells cultured in the presence of SBI and excluding the dye was higher than 80% for the first 2 to 3 days, while their response, again measured by thymidine incorporation, was still inhibited to about 60% by the SBI. Therefore, the presence of the SBI in the culture did not affect cell viability as assayed by Trypan Blue exclusion test while it still inhibited the response to mitogens.

B. Ovalbumin and boiled SBI

As another control, ovalbumin (since it has roughly the same molecular weight as SBI) was often included in the culture to see if it were only the mere presence of a neutral (no anti-proteolytic activity) protein that caused inhibition. Ovalbumin at the same concentration as the inhibitor had no inhibitory effects. Similarly, the boiled SBI inhibitor showed no inhibitory effects on the lymphocyte transformation.

C. Purity of the SBI preparation

The purity of the SBI preparation was also analysed. The analysis of commercial preparations of SBI on normal polyacrylamide gels revealed only one band compared to SDS polyacrylamide gels which revealed two bands; the major one corresponding to the expected molecular mass of the SBI (Kunitz). The minor band had a lower MW (15,000) and could represent a real contaminant or simply a degradation product of the SDS electrophoresis as indicated by the following facts.

- Analysis by analytical ultracentrifugation done with up to 5 mg/ml of SBI revealed only one sedimentation peak.

- Prolonged dialysis of the SBI did not affect its ability to inhibit blast transformation.

- Other commercial preparation of SBI were also very effective in inhibiting transformation.

D. Effect of other protein protease inhibitors

As a further test to verify the specificity of the SBI preparation in inhibiting the lymphocyte blast transformation, the action of similar trypsin inhibitors was also investigated. Figure 4 shows the effect of LBI on the lymphocyte transformation. The inhibition seen here (about 80% at a concentration of 3 mg/ml) is similar to the one caused by the SBI. The effect of another protein inhibitor ovomucoid was also investigated (Table 8).

Although this trypsin inhibitor does not inhibit lymphocyte transformation as well as the other ones, it is still clear that there is significantly less incorporation of thymidine by the stimulated cells in contact with the OM than by the stimulated control cells. Therefore considering all these factors, it can safely be said that it is the anti proteolytic activity of the SBI inhibitor which is active in inhibiting lymphocyte blast transformation.

Figure 4: Effect of LBI on LAG stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with LBI and mitogen on day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no LBI).

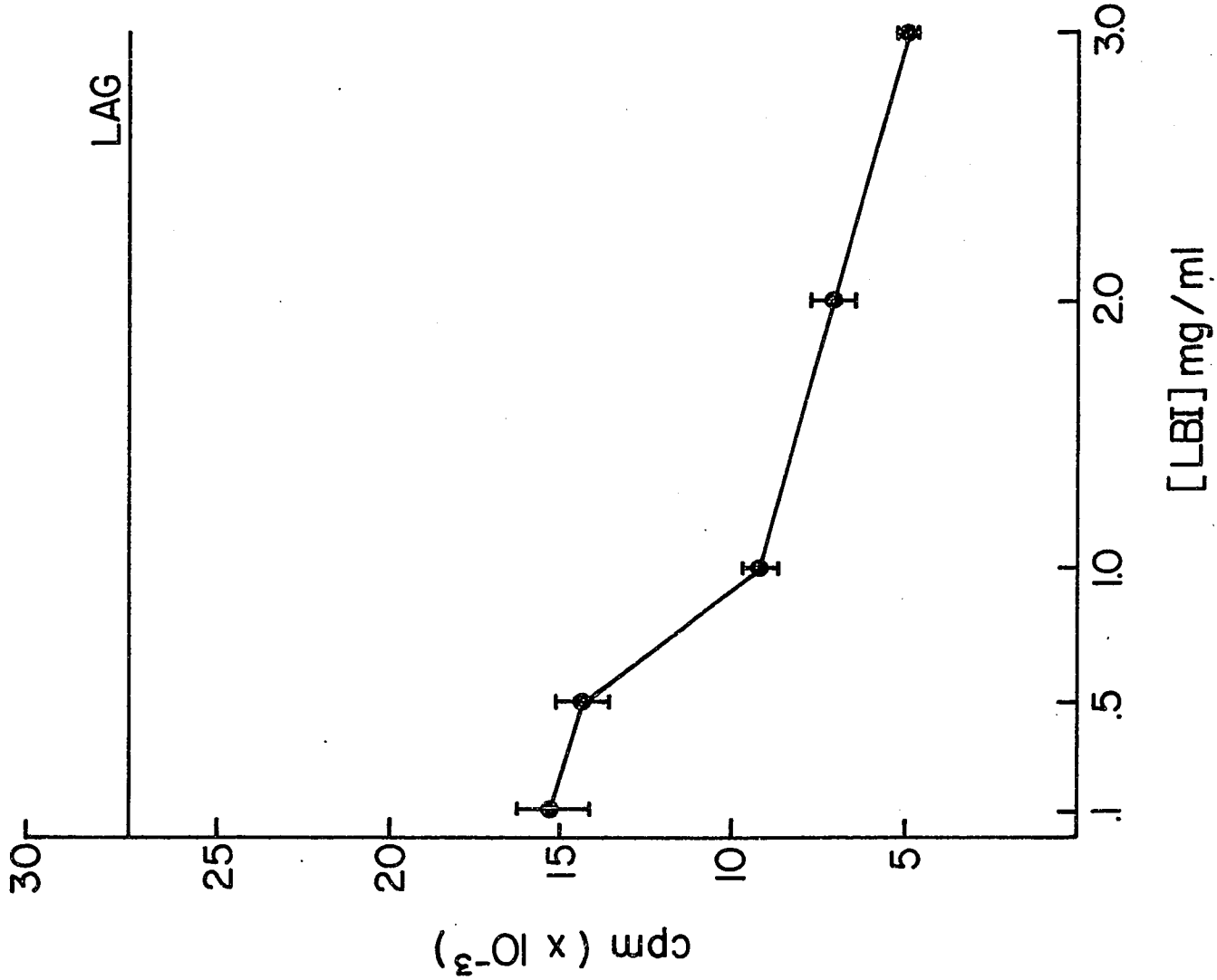

LAG
30
25
20
15
10
5
cpm (x 10^{-3})
.1
.5
1.0
2.0
3.0
[LBI] mg/ml

TABLE 8

Effect of OM on Stimulation of Human Lymphocytes by LAG

Concentration of OM (ug/ml)

	0	250	500	1000	2000	3000
	29,324*		21,807	23,996	20,310	17,364
A	1,460**		1,287	2,254	2,076	1,022
			(26%)***	(18%)	(31%)	(41%)
	28,642	24,412	22,820			
B	400	1,651	8,425			
		(15%)	(20%)			
	17,318	14,292	8,540		11,000	
C	2,208	838	739		948	
		(17%)	(50%)		(36%)	

* ^{3}H-thymidine incorporation in cultures harvested on day 3, expressed as cpm.

** Standard deviation of the four replicates expressed as cpm.

*** % of inhibition of the mitogen response.

V. EFFECTS OF SBI ON MOUSE CELLS

It was of interest to know whether only human lymphocytes were affected by the SBI. Figure 5 shows that incorporation of ^{3}H Thymidine into DNA of stimulated mouse splenocytes was also inhibited by the presence of SBI in the culture medium. Thus the inhibitor seems also to be affecting, in roughly the same way (more than 80% inhibition of the blast transformation at 3 mg/ml of SBI), the mouse lymphocytes in which the trypsin stimulating effect was first discovered (5, 6).

VI. EFFECT OF SBI ON URIDINE INCORPORATION

It was also interesting to see whether only DNA synthesis was affected by the SBI or if other parameters measuring blast transformation were also affected. Table 9 shows that incorporation of ^{3}H uridine, presumably into RNA of stimulated human and mouse cells, was also sensitive to the SBI.

It therefore seems that the anti-trypsin activity of the SBI inhibitor can inhibit the lymphocyte blast transformation, induced by different mitogens and agents, as measured by two different parameters (thymidine and uridine incorporation. This inhibition was seen with human as well as with mouse lymphocytes.

Figure 5: Effect of SBI on ConA stimulation of mouse lymphocytes.

^{3}H-thymidine incorporation in cultures treated with SBI and mitogen at day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no SBI).

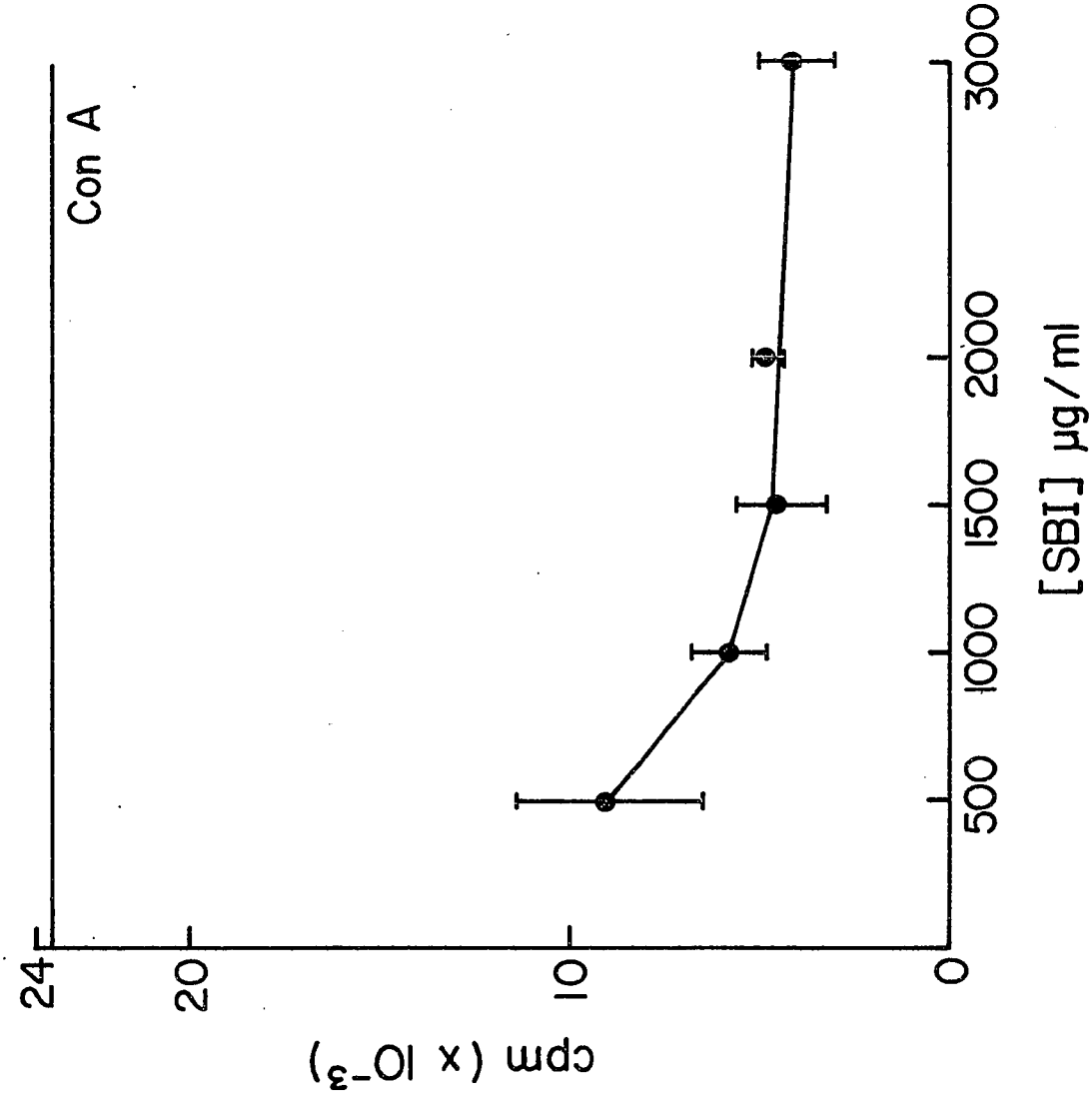

Con A
24
20
10
0
cpm (x 10⁻³)
500
1000
1500
2000
3000
[SBI] µg/ml

TABLE 9

Effect of SBI on Uridine Incorporation of Human Lymphocytes

Concentration of SBI (ug/ml)			
0	500	1000	3000
Expt. 1			
51,084*		22,768	
3,000**		2,870	
		(55%)***	
Expt. 2			
21,730	19,344		7,665
1,160	903		602
	(11%)		(76%)

* ^{3}H-uridine incorporation by cultures harvested on day 3, expressed as cpm.

** Standard deviation of the four replicates expressed as cpm.

*** % inhibition of the mitogen response.

VII. EFFECT OF SYNTHETIC INHIBITORS

It was of some interest to discover whether the effect of SBI or protein protease inhibitors in general was specific to these inhibitors or whether other inhibitors had similar effects. Darzynkiewicz and Arnason (64) had already found that TLCK and TPCK at concentration ranging from 10^{-7} to 10^{-4} M caused the suppression of RNA synthesis (as measured by uridine incorporation). Figures 7.1 and 7.2 show the effects of TLCK on the incorporation of ^{3}H thymidine in human lymphocytes stimulated by different agents, while Figure 7.3 shows the effect of TPCK. Therefore, in my culture system, TLCK and TPCK inhibition of lymphocyte transformation as measured by thymidine incorporation was comparable to that found by by Darzynkiewics and Arnason on PWM stimulated cells (64) TLCK gave almost 100% inhibition at 10^{-4} M while TPCK gave similar inhibition at 10^{-5}M. PMSF was also tried in the human cultures at concentration ranging from 10^{-6} to 10^{-4}M and results are shown in Figure 7.4.

VIII. EFFECT OF TLCK ON STIMULATED MOUSE CELLS

Mouse cells were also affected by TLCK as shown in Figure 8. Here, almost 100% inhibition was achieved at concentration of 2.5 X 10^{-5}M, eight times less than what is required to achieve similar inhibition with human cells.

Figure 7.1: Effect of TLCK on mitogen stimulation of human lymphocytes.

Percentage of inhibition of mitogen stimulation (measured by ^{3}H-thymidine incorporation) caused by TLCK. ConA (▲—▲), PHA (■—■), LAG (●—●). Cultures were treated by the mitogens and TLCK on day 0 and harvested on day 3.

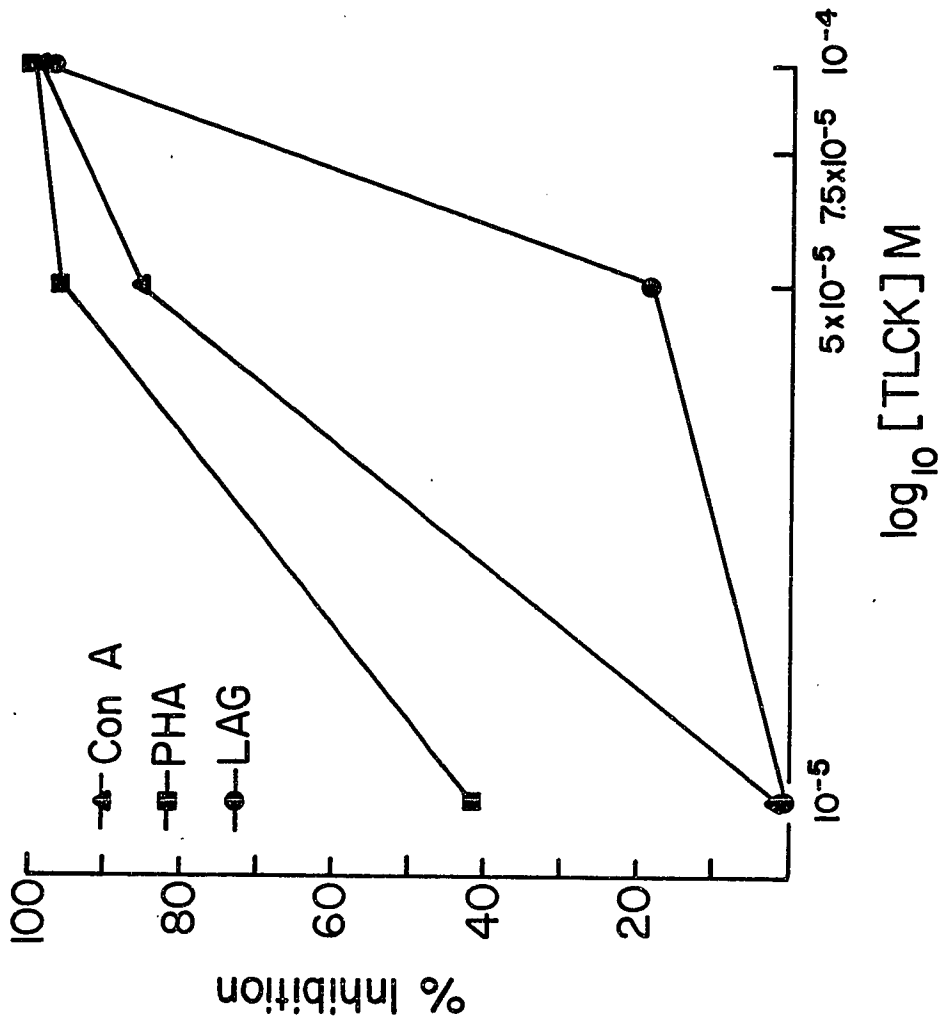

% Inhibition
100
80
60
40
20
Con A
PHA
LAG
10^{-5}
5×10^{-5}
7.5×10^{-5}
10^{-4}
$\log_{10}$ [TLCK] M

Figure 7.2: Effect of TLCK on IO_4^- stimulation and on MLR of human cells.

Percentage of inhibition of IO_4^- stimulation caused by TLCK, (●—●). IO_4^- and TLCK were added on day 0, and cultures were harvested on day 3. (▲—▲) shows the effect of TLCK in the MLR. TLCK and cells were mixed on day 0 and the cultures were harvested on day 5. Response was measured by ^3H-thymidine incorporation in both cases.

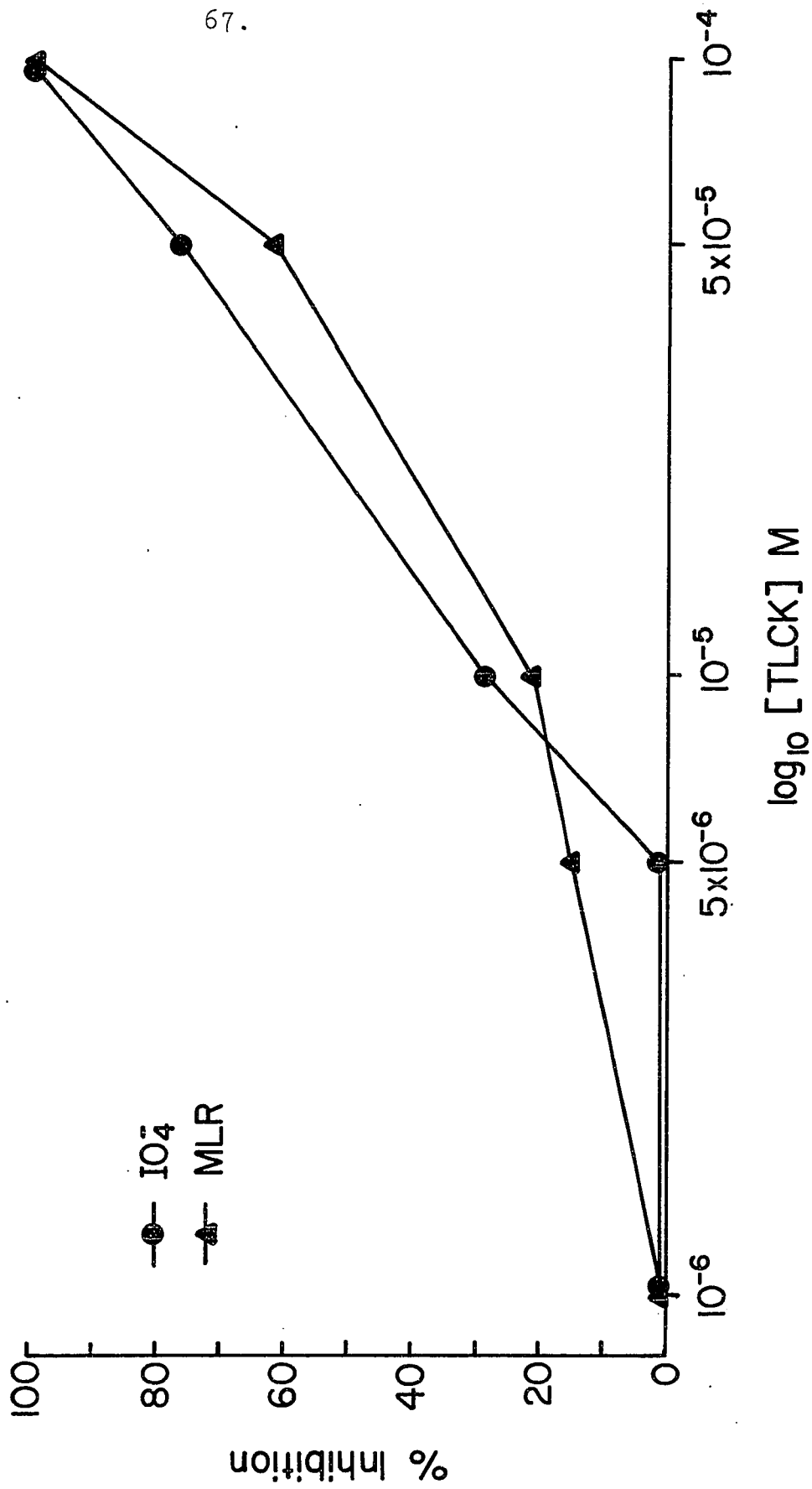

% Inhibition
100
80
60
40
20
0
10^{-6}
5×10^{-6}
10^{-5}
5×10^{-5}
10^{-4}
$\log_{10}$ [TLCK] M
IO_4^-
MLR

Figure 7.3: Effect of TPCK on mitogen stimulation and on MLR of human cells.

Percentage of inhibition of the response to mitogens Con A (—●—●—), LAG (—■—■—), as measured by ^{3}H-thymidine incorporation, of TPCK treated cell cultures. TPCK and mitogens were added on day 0 and cultures were harvested on day 3. For the MLR (—▲—▲—), cells + TPCK were mixed on day 0 and harvested on day 5.

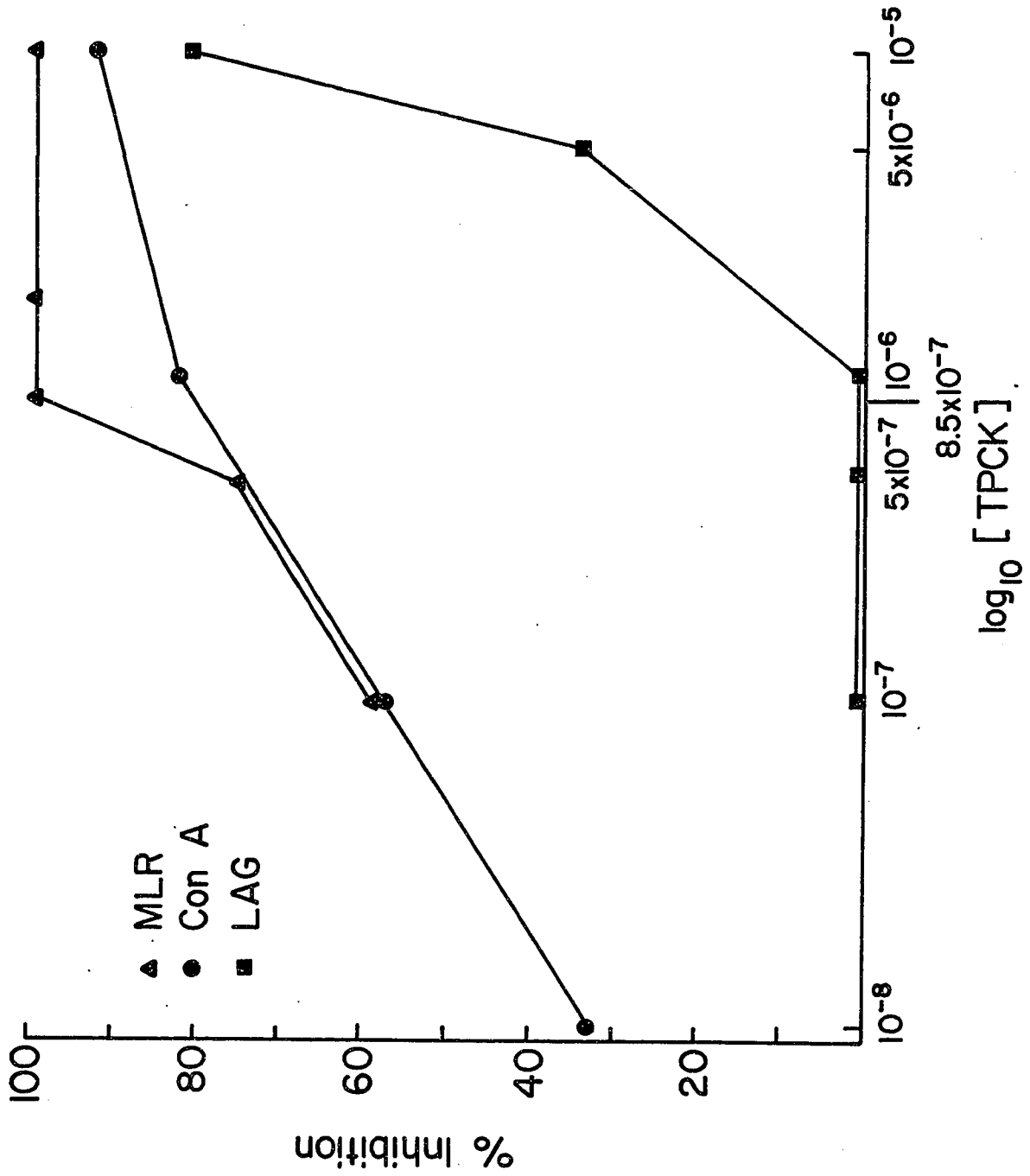

% Inhibition
100
80
60
40
20
MLR
Con A
LAG
10^{-8}
10^{-7}
5×10^{-7}
8.5×10^{-7}
10^{-6}
5×10^{-6}
10^{-5}
$\log_{10}$ [TPCK]

Figure 7.4: Effect of PMSF on LAG stimulation and on MLR of human cells.

Percentage of inhibition of LAG stimulation caused by PMSF (●—●). LAG and PMSF were added on day 0 and cultures were harvested on day 3. (▲—▲) shows the effect of PMSF in the MLR. PMSF and cells were mixed on day 0 and the cultures were harvested on day 5. Response was measured by ^{3}H-thymidine incorporation in both cases.

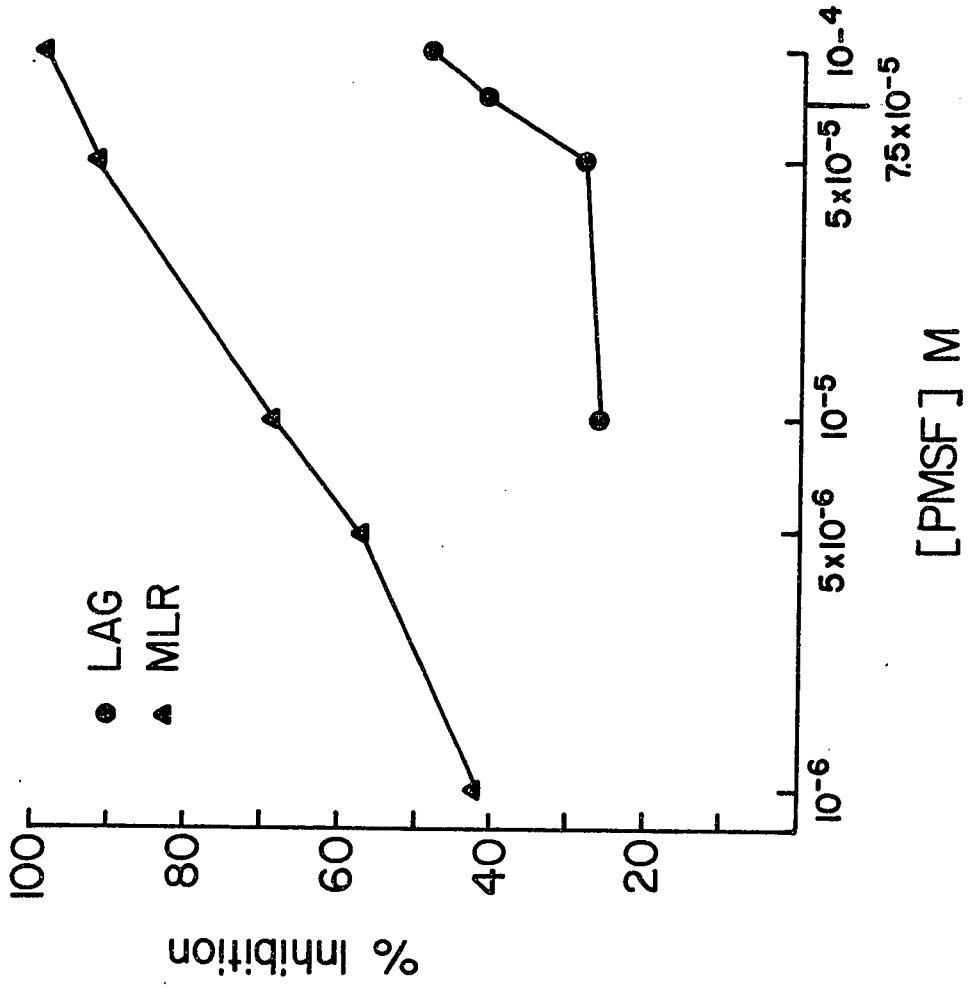
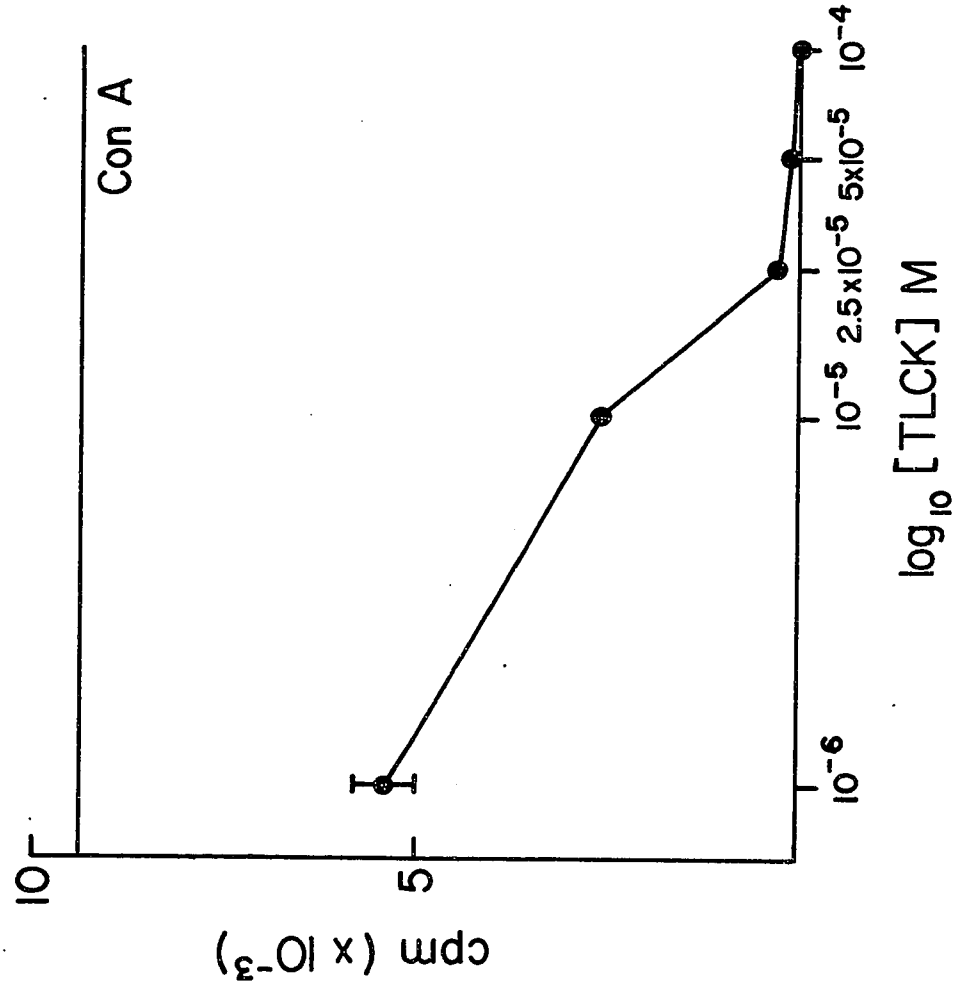

% Inhibition
100
80
60
40
20
LAG
MLR
10⁻⁶
5x10⁻⁶
10⁻⁵
5x10⁻⁵
7.5x10⁻⁵
10⁻⁴
[PMSF] M

Figure 8: Effect of TLCK on ConA stimulated mouse lymphocytes.

^{3}H-thymidine incorporation in cultures treated with TLCK and mitogen on day 0, harvested on day 3. The upper horizontal line represents the average incorporation in stimulated controls (no TLCK).


Con A
10
5
cpm (x 10^-3)
10^-6
10^-5
2.5x10^-5
5x10^-5
10^-4
log10 [TLCK] M

IX. EFFECT OF TLCK ON URIDINE INCORPORATION

As for SBI, TLCK also inhibits uridine incorporation by stimulated mouse cells as shown in Table 10.

TABLE 10

Effect of TLCK on Incorporation of Uridine of Mouse Transformed Lymphocytes

	Concentration of TLCK (M)						
	0	10^{-6}	10^{-5}	2.5×10^{-5}	5×10^{-5}	7.5×10^{-5}	10^{-4}
A	20,968						189 19 (99%)
B	78,540*	50,267	33,580	11,080	2,636	493	1,000
	13,700**	5,800	3,870	650	266	98	864
		(36%)***	(57%)	(86%)	(97%)	(100%)	(100%)
C	21,410	12,430	12,184	6,530	2,650		205
	4,456	3,300	3,355	2,290	969		55
		(42%)	(43%)	(70%)	(88%)		(99%)

* ^{3}H-uridine incorporation by cultures stimulated by ConA and harvested on day 3, expressed as cpm.

** Standard deviation of the four replicates expressed as cpm.

*** % inhibition of the mitogen response.

X. VIABILITY STUDIES

Viability studies of cells exposed to different synthetic inhibitors were performed using the Trypan Blue exclusion test. The viability of cells was not impaired by up to 10^{-4} M of TLCK and PMSF and by up to 10^{-5} M of TPCK. Overnight dialysis of the TLCK preparation did not change in any way its inhibitory effect.

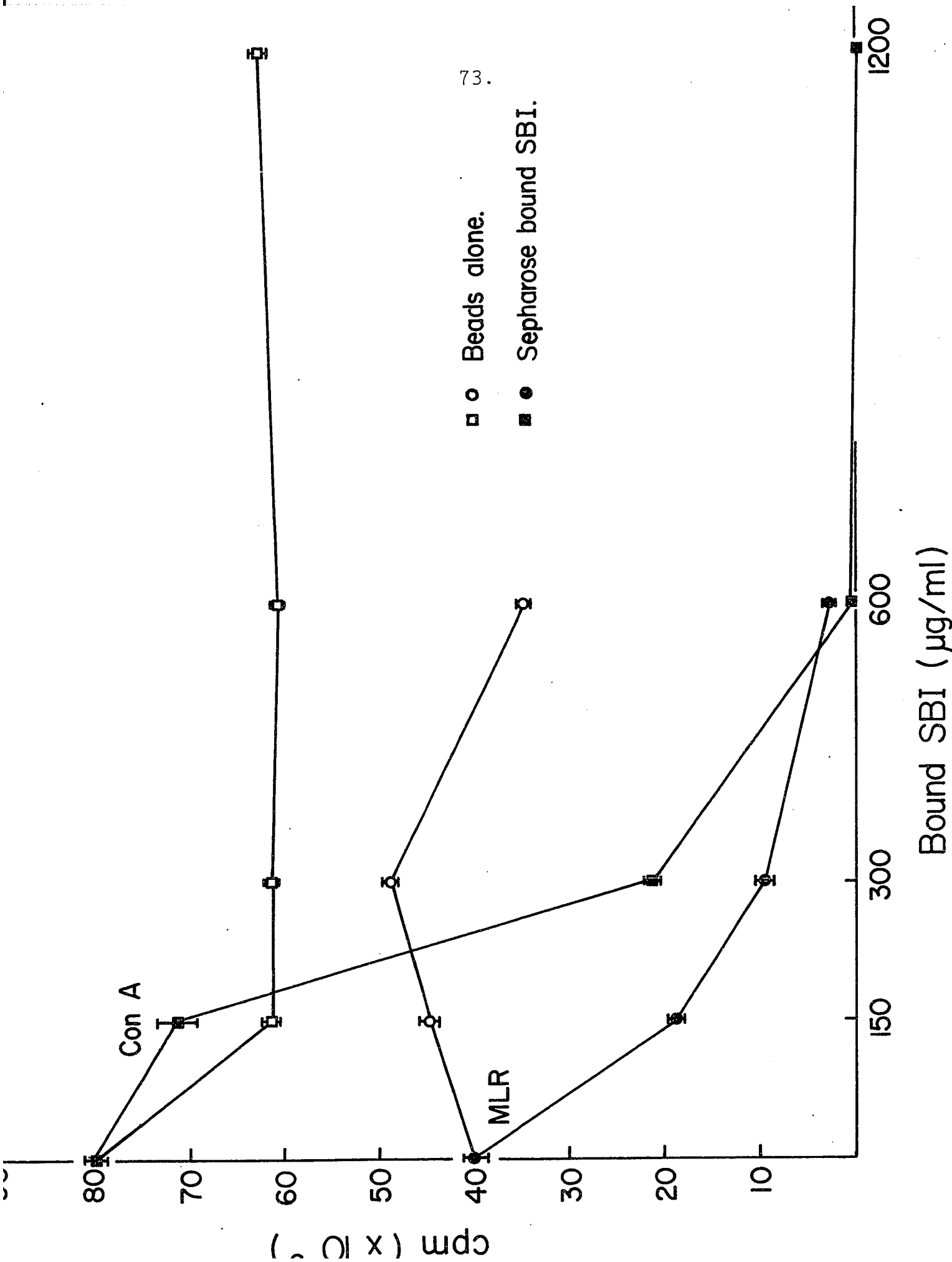
Thus both protein and synthetic inhibitors inhibit blast transformation of both human and mouse lymphocytes as measured by the two different parameters. It therefore seems likely that there is a proteolytic step essential to lymphocyte blast transformation.

XI. EFFECT OF INSOLUBLE SBI ON LYMPHOCYTES TRANSFORMATION

Now that the presence of an "essential" proteolytic step had been confirmed, it was interesting to investigate whether or not this proteolytic step took place at the surface or inside the cells. Figure 11 shows the effects of SBI covalently bound to Sepharose beads on transformation of lymphocytes. It is clearly seen that Sepharose-bound SBI has a strong inhibitory effect on Con A and MLR stimulation. Sepharose beads without cross-linked SBI had virtually no inhibitory action. Thus, both forms of stimulation could be inhibited almost completely by the covalently bound SBI.

Figure 11: Effect of SBI covalently bound to Sepharose beads on ConA stimulation and on MLR of human cells.

^{3}H-thymidine incorporation in cultures treated with Sepharose beads alone and Con A (⊟—⊟), Sepharose bound SBI and Con A (■—■), Sepharose beads alone in MLR (○—○), and Sepharose bound SBI in MLR (●—●). The beads and/or the inhibitor and the ConA were added on day 0 and cultures were harvested on day 3 while in the MLR, the treated cells were mixed at day 0 and harvested on day 5. It is noteworthy that beads alone (no SBI) did not cause significant inhibition.


Beads alone.
Sepharose bound SBI.
Con A
MLR
cpm (x 10³)
80
70
60
50
40
30
20
10
150
300
600
1200
Bound SBI (µg/ml)

XII. KINETIC STUDIES OF THE ACTION OF SBI AND TLCK

At which point after stimulation, did the inhibitor act? Kinetic studies of the action of different protease inhibitors were done in order to answer this question. Figure 12.1 shows the effect of SBI when added at the same time as the stimulating agent or 1,2,3,4,20 or 24 hours after. When the inhibitor is added 4 hours after the mitogens, there is virtually no inhibition whatsoever and when addition is after 20 hours, there was no inhibition noted. It is noteworthy that when the inhibitor was added two hours before the stimulating agent, there was no significant difference in the inhibition compared with the simultaneous addition of the inhibitor and the mitogens. Figure 12.2 shows the results of the same experiment using TLCK (concentration of 7.5×10^{-5} M) instead of SBI. It is noteworthy here that addition of TLCK 24 hours after the mitogens still produced 35% inhibition but there was no inhibition when TLCK was added 48 hours after Con A. Figure 12.3 shows the result of the same experiment this time using mouse splenocytes instead of human lymphocytes. Similar results were obtained.

Figure 12.4 shows the results of three different experiments using three different inhibitors. Here the stimulated cells were cultured in one large flask and except for the control cells, were all exposed to the inhibitor at time zero. After three hours, three different

Figure 12.1: Effect of SBI (3 mg/ml) added at various times on LAG stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with the mitogen (LAG) at time 0 and with SBI at various time after LAG. The cultures were harvested on day 3. The upper horizontal line represents the average incorporation in stimulated cultures (no SBI).

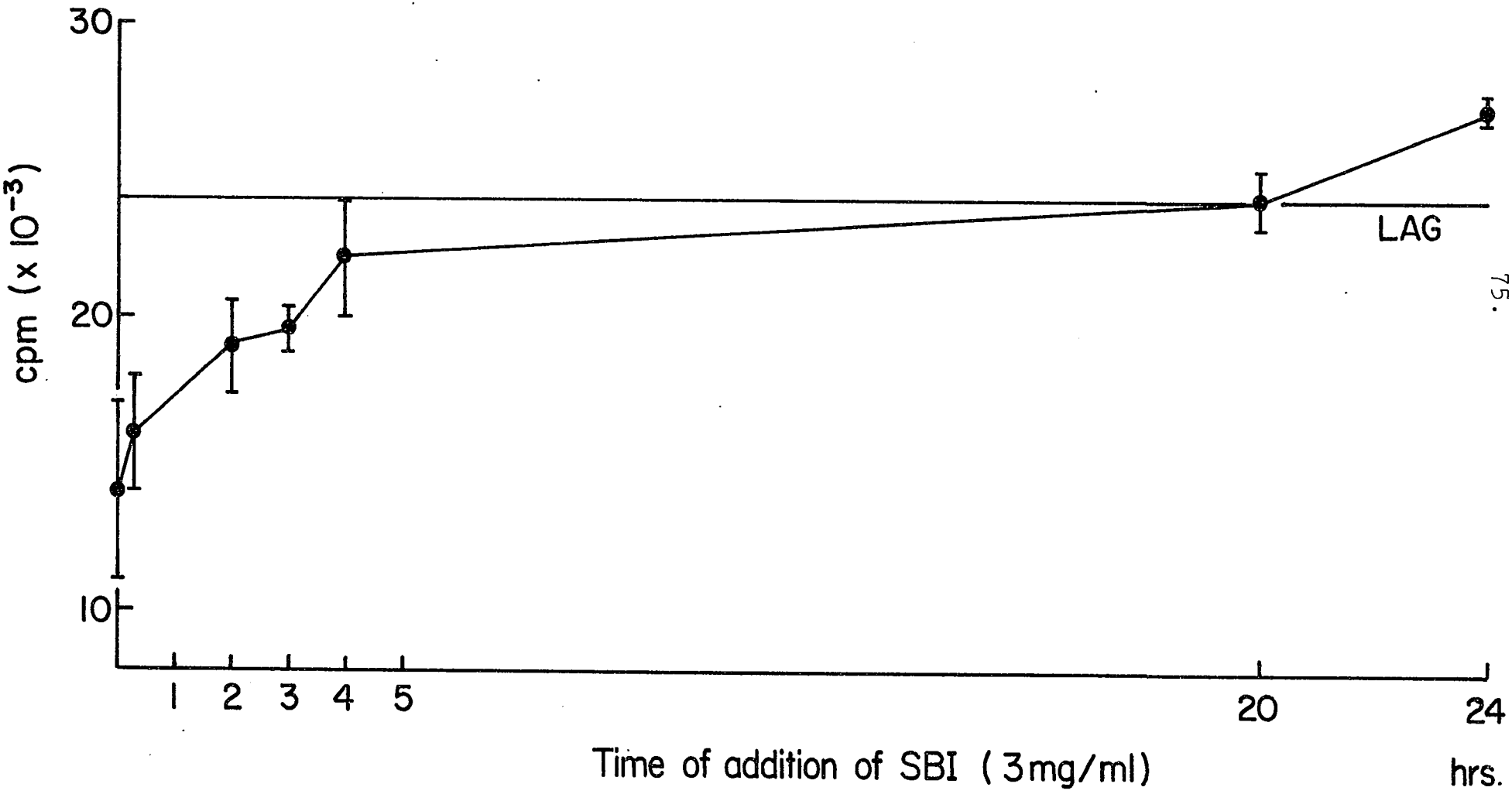

30
20
10
cpm (x 10⁻³)
LAG
1
2
3
4
5
20
24
Time of addition of SBI (3mg/ml)
hrs.

Figure 12.2: Effect of TLCK added at various times on ConA stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures treated with the mitogen (ConA) at time 0 and with TLCK ($7.5 \times 10^{-5}M$) at various time after ConA. The cultures were harvested on day 3. The upper horizontal line represents the average incorporation in stimulated cultures (no SBI).

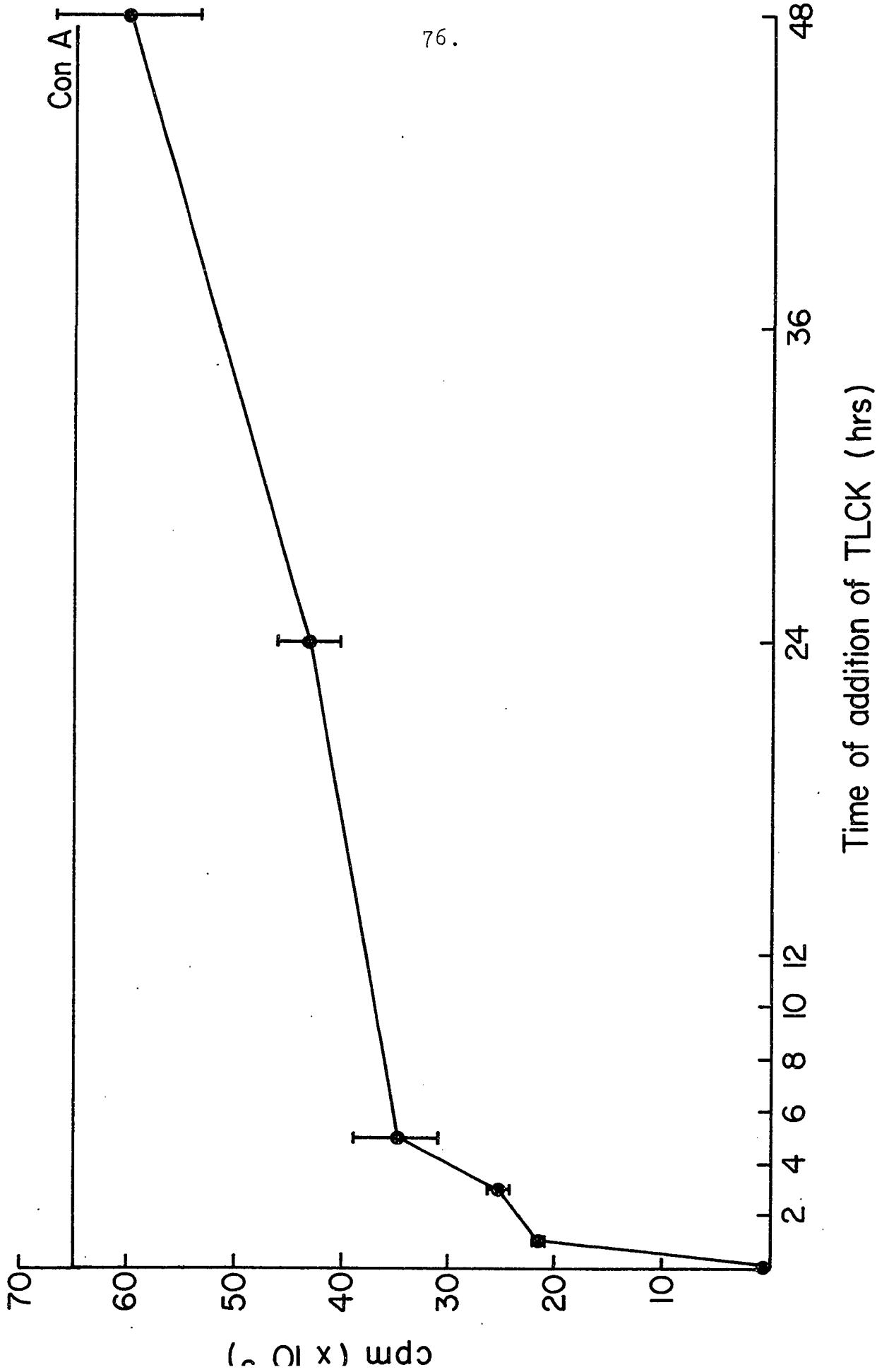

Con A
70
60
50
40
30
20
10
cpm (x 10³)
2
4
6
8
10
12
24
36
48
Time of addition of TLCK (hrs)

Figure 12.3: Effect of TLCK, added at various times on ConA stimulation of mouse lymphocytes.

^{3}H-thymidine incorporation in cultures treated with the mitogen (ConA) at time 0 and with TLCK (7.5×10^{-5}M) at various time after ConA. The cultures were harvested on day 3. The upper horizontal line represents the average incorporation in stimulated cultures (no SBI).

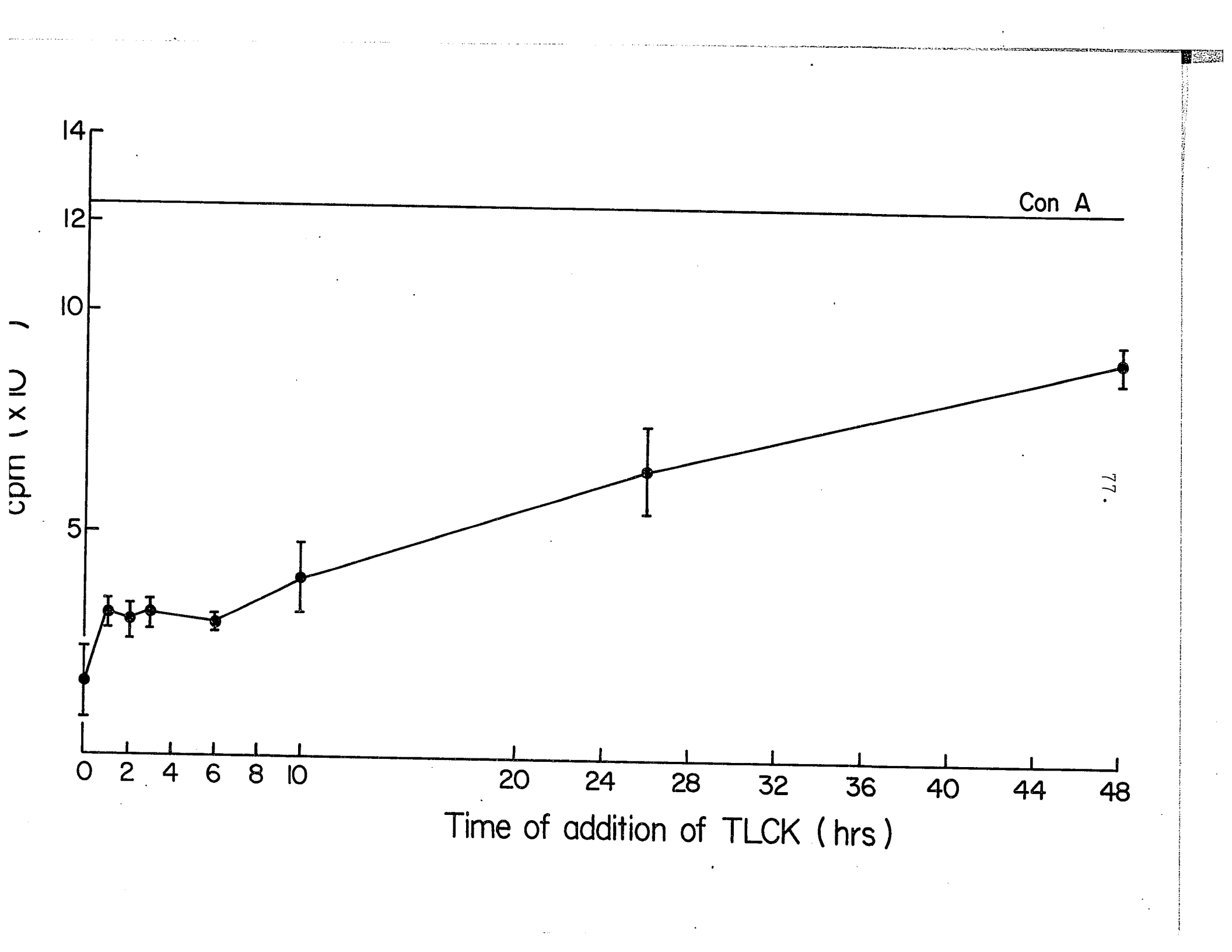
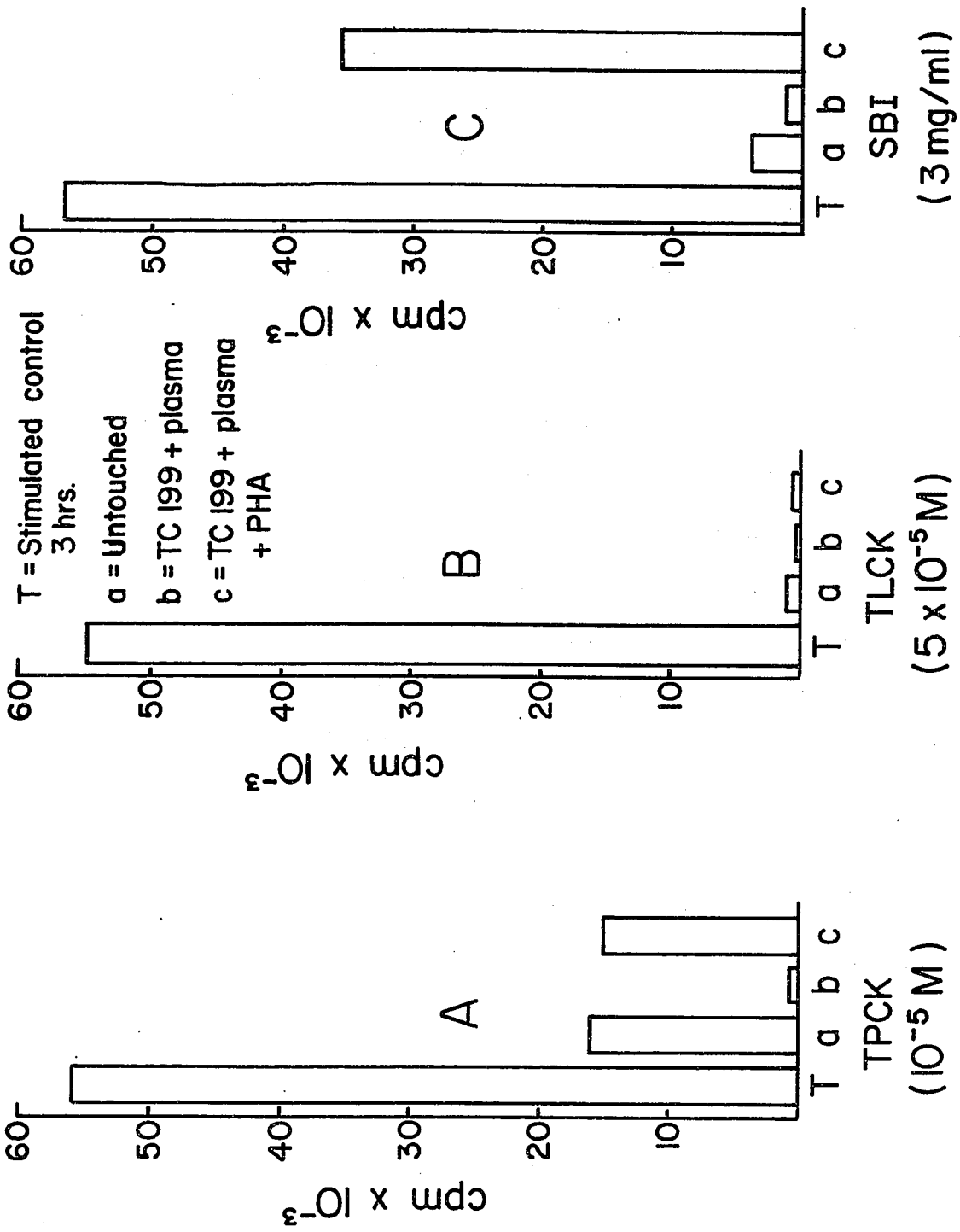

14
12
10
5
Con A
cpm (x10)
0 2 4 6 8 10 20 24 28 32 36 40 44 48
Time of addition of TLCK (hrs)

Figure 12.4: Effect of exposure to protease inhibitors for 3 hours on PHA stimulation of human cells.

^{3}H-thymidine incorporation in cultures treated with PHA and TPCK (10^{-5}M), TLCK (5×10^{-5}M) and SBI (3 mg/ml) on time 0 then washed 3 hours after and reincubated in mitogen-free, inhibitor-free medium (b) or in inhibitor-free medium (c). Incorporation in cultures having had mitogen but no inhibitor and no washing is shown (T) along with cultures having had PHA and inhibitors but no washing (a).


T = Stimulated control 3 hrs.
a = Untouched
b = TC 199 + plasma
c = TC 199 + plasma + PHA
cpm x 10⁻³
60
50
40
30
20
10
A
B
C
T a b c
TPCK (10⁻⁵ M)
TLCK (5 x 10⁻⁵ M)
SBI (3 mg/ml)

aliquots were taken; aliquot (a) was put directly into the culture plate; the second aliquot (b) was centrifuged, the cells were washed and resuspended in fresh medium and then added to the culture plates. The third aliquot (c) was treated exactly like (b) except that the fresh medium, added after the cells were washed, contained fresh PHA. Everything was then harvested at 72 hours. Therefore, in the first aliquot, cells were in contact with the inhibitor and the mitogen for 72 hours. In the second one, the cells were in contact with the inhibitor for 72 hours but only three hours in contact with the mitogen. In the third aliquot, the cells were in contact with the mitogens for 72 hours and only three hours with the inhibitor. As for TPCK and TLCK, there is no difference between (a) and (c) indicating that the cells were already inhibited in the first three hours and since these are irreversible proteolytic inhibitors, even washing the cells and adding fresh mitogens did not permit the blast transformation. SBI, on the other hand, is a reversible inhibitor and it can clearly be seen that more than 50% of the stimulation is recovered when the cells are washed after three hours of contact with the inhibitor and the mitogen and resuspended in medium containing fresh mitogens. This was expected since the SBI is a reversible inhibitor and therefore the washing step resulted in the removal of any bound or free SBI. The fact that

(b) in all three experiments show no stimulation was also expected since these cells were in contact with PHA for only three hours and it was shown that they must remain in contact for at least 18 hours to be stimulated. This control was included to see if the inhibitor alone could cause an unspecific stimulation of the cells. These results also tend to show that SBI does not drastically alter the viability of the cells, since the response can be recovered after the washing out of the inhibitor. Thus this last experiment along with the kinetic experiment tends to show that the hypothesized proteolytic step is an early event after stimulation and that if it is inhibited no lymphocyte stimulation can take place.

XIII. POSSIBLE SOURCE OF THIS PROTEOLYTIC ENZYME

Besides hypothesizing that there was an essential proteolytic event common to all types of lymphocyte blast transformation occurring at the cell membrane, Kaplan and Bona further predicted that the mitogenic effect of the trypsin added to the mouse cells acted by short-circuiting an endogenous protease. However, the data presented until now do not discriminate among the several possible sources of this proteolytic enzyme. Rather than being produced by the lymphocytes themselves, the hypothesized enzyme could very well be already present in the plasma or it could be produced by other cells present in the cultures. In order

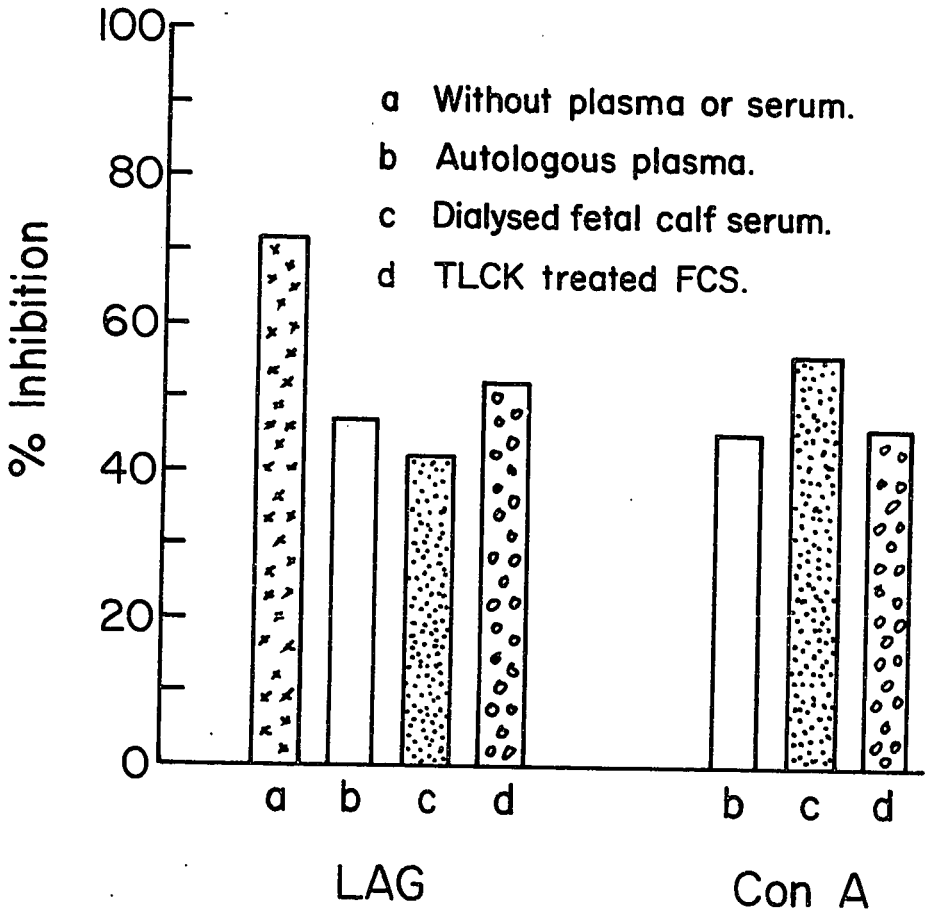
to investigate these different possibilities the following experiments were performed.

A. Role of the Plasma

One possible source of this enzyme is certainly the plasma since this component of blood is known to contain a multitude of enzymes and is always present in the culture medium. Figure 13.1 shows the percentage of inhibition of the lymphocyte blast transformation obtained with varying supplements of plasma, serum or treated serum. Except for the different plasma, the protocol is similar to the other experiments. More than 70% of the inhibition occurred when no plasma was present in the culture. Since it is very difficult to culture human lymphocytes without plasma or serum, good mitogenic stimulation was achieved in only a few experiments. However, when good stimulation occurred, SBI always had a high inhibitory effect. There was no significant difference in the response of cells cultured withFCS instead of autologous plasma or when the culture medium was supplemented with TLCK pre-treated FCS. Prolonged dialysis of the FCS before being added to the culture did not affect the results. Also, experiments show that if autologous serum is used instead of plasma, there is also no significant difference in the resulting inhibition.

Figure 13.1: Effect of the plasma on the SBI inhibition of stimulation of human lymphocytes.

Percentage of inhibition of LAG and ConA stimulation caused by SBI (1 mg/ml) when the culture medium is supplemented with: (a) no plasma or serum, (b) autologous plasma, (c) dialysed FCS and (d) TLCK-Pre-treated FCS. The cultures were treated with SBI and the mitogen on day 0 and harvested on day 3.


100
80
60
40
20
0
% Inhibition
a Without plasma or serum.
b Autologous plasma.
c Dialysed fetal calf serum.
d TLCK treated FCS.
a b c d
b c d
LAG
Con A

B. Role of the Macrophages

The second obvious possibility to investigate was the macrophages. As mentioned before in the Introduction, macrophages contain a multitude of proteolytic enzymes which could certainly take part in the triggering of lymphocyte blast transformation. Use of different purification methods (see Materials and Methods) to deplete the culture of macrophages gave no positive results. The mitogen response was never decreased in the purified culture while the antigen response seemed to be decreasing but showed quite a bit of variability in the results. Therefore, no useful data were obtained from these experiments and the macrophages could not be excluded as a possible source of the proteolytic enzyme.

C. Role of the other cells present in the culture

Figure 13.2 shows the effect of the purification method on the response to LAG. Figure 13.3 shows the different percentage of inhibition obtained, depending on the purification method. There is less inhibition when the inhibitor is added to the Ficoll Hypaque purified cultures.

Figure 13.2: Effect of purification method on LAG stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures of lymphocytes purified on Ficoll Hypaque (A) or on Dextran (B) stimulated by LAG. Incorporation into control cultures, having had no LAG is also shown (T). Cultures were harvested on day 3.

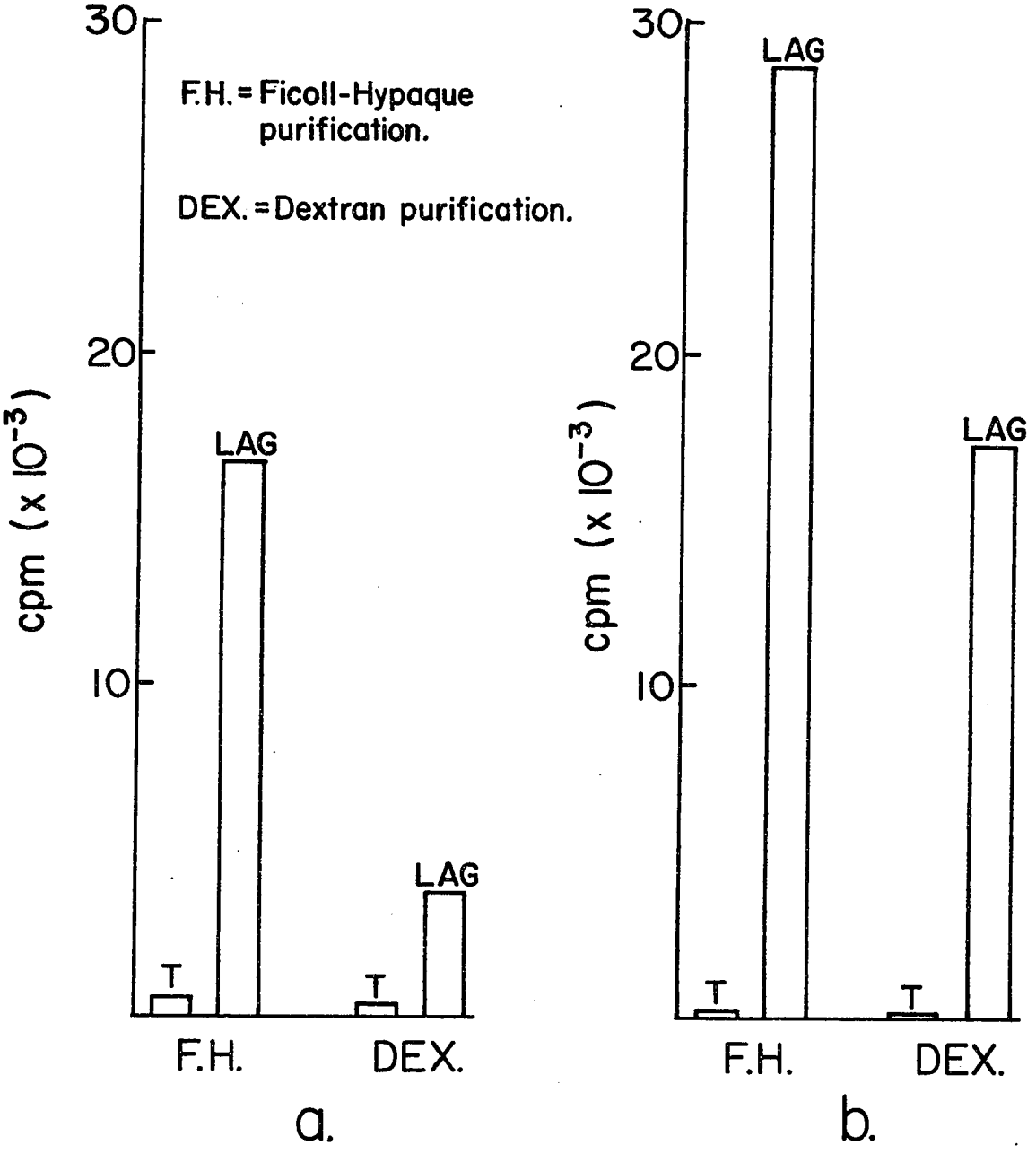
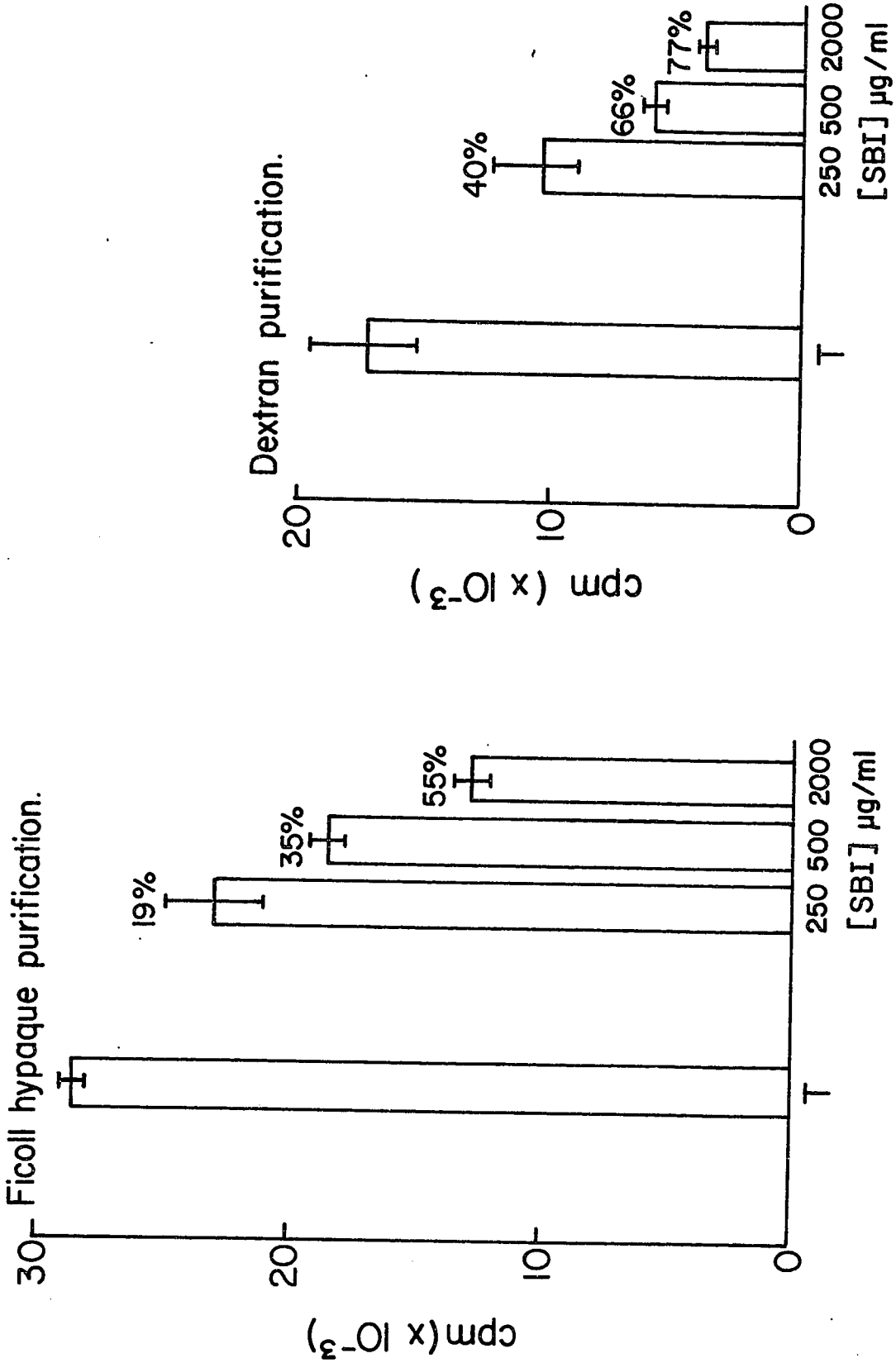

30
20
10
cpm (x 10⁻³)
F.H. = Ficoll-Hypaque purification.
DEX. = Dextran purification.
LAG
LAG
T
T
F.H.
DEX.
a.
30
20
10
cpm (x 10⁻³)
LAG
LAG
T
T
F.H.
DEX.
b.

Figure 13.3: Effect of the purification method on the SBI inhibition of LAG stimulation of human lymphocytes.

^{3}H-thymidine incorporation in cultures (purified by different method) treated with LAG and SBI on day 0 and harvested on day 3. The percentage of inhibition for each concentration of SBI is indicated. Incorporation into control cultures (no SBI) is also shown (T).


Ficoll hypaque purification.
cpm (x 10⁻³)
30
20
10
0
T
19%
35%
55%
250 500 2000
[SBI] µg/ml
Dextran purification.
cpm (x 10⁻³)
20
10
0
T
40%
66%
77%
250 500 2000
[SBI] µg/ml

D I S C U S S I O N S and C O N C L U S I O N

This work was begun with the intention of verifying Kaplan's and Bona's hypothesis which predicted that there was one or more than one endogenous protease whose action at the cell membrane was essential to the subsequent events of blast transformation (6). There were different ways to test this hypothesis but the simplest approach seemed to be the use of protease inhibitors.

Because Kaplan and Bona were able to stimulate mouse lymphocytes with trypsin, it was thought that the hypothesized enzyme could itself be a trypsin or trypsin-like enzyme. It was thus assumed that when Kaplan and Bona added trypsin to the culture medium, they, in fact, "short circuited" the endogenous essential enzyme. Protease inhibitors, particularly SBI were then added to different cultures stimulated by a variety of agents.

Soon after the first experiment, it became evident that SBI at a concentration ranging from 250 ug/ml to 3000 ug/ml could block up to 80% of thymidine incorporation in the stimulated human cells. It was interesting to note that SBI inhibited blast transformation induced by various agents including 10ɳ and MLR acting in different ways to cause lymphocyte transformation. SBI then, seemed to inhibit a proteolytic event common to all these

stimulating mechanisms. When uridine incorporation was used as another parameter to measure blastogenesis, the addition of SBI to the cultures still produced the same inhibitory effect.

It was also interesting to note that SBI caused the same inhibition of lymphocyte transformation when added to mouse cells stimulated by different mitogens. Therefore, the essential proteolytic step inhibited by SBI is not only related to human cells but is also found in mouse cells. This result was expected since it is with mouse cells that the mitogenic effect of trypsin was first discovered. It is noteworthy that, although we were the first ones, we were not alone in reporting the inhibitory effect of SBI on lymphocyte transformation (65) and mouse cells. A few years ago, Kast (66) and Vosiga (67) briefly reported in abstracts the inhibitory effect of SBI on human lymphocyte transformation. Recently, Hart and Streilein working with hamster cells (68) and Algon et al, working with human cells (69), also reported the inhibitory effect of SBI. However, Vischer (5), Darzynkiewicz et al (64) and Hirschhorn et al (70) failed to observe an inhibition of mitogen inducedblastogenesis caused by SBI. There is no explanation for this discrepancy. We think that our results are legitimate since viability studies along with different

purification procedures (gel electrophoresis, ultra-centrifugation analysis) were done with SBI and show that the inhibitory effect was not an artifact due to cell death or to a contaminant of the SBI preparation. In addition, OV was often used as a control protein in cultures and although there was sometimes a small inhibition (10-15%) at the highest concentration, it usually had no inhibitory effect indicating no unspecific effect of proteins. The inhibition caused by LBI and OM (showing similar anti-proteolytic activity) at roughly the same concentration might suggest that it is the anti-proteolytic activity of these agents that is causing the inhibition of transformation, and not any other non specific action.

The inhibitory effect of synthetic inhibitors has been reported by some authors who obtained similar results to ours. Darzynkiewicz and Arnason reported the inhibitory action of TPCK, TLCK, BTEE, and TAME (64) while Saito et al (71) showed the inhibitory effect of leupeptin (a tripeptide protease inhibitor) and antipain (a microbial protease inhibitor). Recently, Grazel et al (72) and Hart et al (68) reported the inhibition of mitogen response by TLCK. Hirschhorn et al also reported the inhibitory action of EACA but at concentration up to $5X10^{-2}M$ (70). Although the specificity of the action of EACA at these concentrations can be discussed, nevertheless, we were able to confirm these findings. In addition, we have found that PMSF at

concentration of 5×10^{-5} to 10^{-4} M has a strong inhibitory effect. It is noteworthy that Hart et al failed to note the inhibitory effect of PMSF (68); however, their experiments were done with hamster cells and this is probably the source of discrepancy.

Unlike the protein protease inhibitors, the chloromethyl ketone protease inhibitors owe their specific inhibition of certain enzymes to their ability to alkylate their active site. These inhibitors could also alkylate any other reactive chemical group including free SH groups. However, we noticed along with other authors (64) (68) that TPCK is, on a molar basis, a more potent inhibitor of blast transformation than TLCK. Darzynkiewicz et al have shown that BTEE (a chymotrypsin substrate) was more potent than TAME (a trypsin substrate) in suppressing blast transformation. Since these agents have no alkylating properties, the high inhibition produced by BTEE can only be explained by its affinity to chymotrypsin. Thus this observation is in agreement with our results showing that TPCK is more active than TLCK which seems to implicate the anti-protease activity of the inhibitors.

TPCK and TLCK are not intrinsically cytotoxic (at the highest concentration used) as indicated by direct viability studies and as previously reported (64).

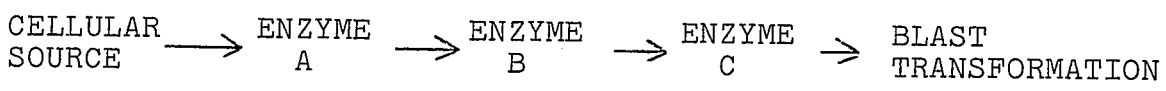
The non-cytotoxic effect was also shown in experiments where lymphocytes were exposed to TLCK (at inhibitory concentrations) for 12-24 hours then washed and resuspended in fresh medium with mitogen. Subsequent blastogenic response was almost like the response of untreated control cells.

All these facts are consistent with the hypothesis of having a proteolytic step as the target of these synthetic agents but no firm conclusions can be drawn on the site of action of these agents since unlike the protein protease inhibitors, the synthetic agents cause inhibition of the basal thymidine and uridine incorporation (incorporation by the resting, non-stimulated control cells).

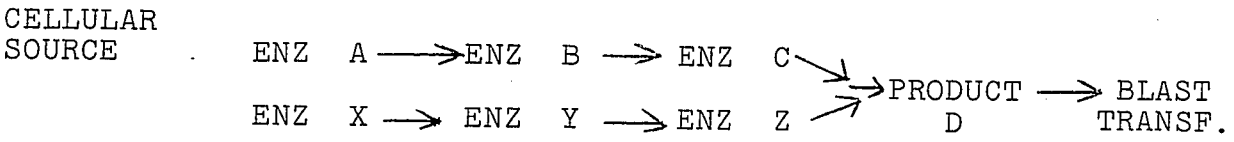
ONE ENZYME?

Since TPCK, a specific chymotrypsin inhibitor, inhibits the blast transformation as well as TLCK and SBI, a specific trypsin inhibitor, it seems that more than one enzyme is involved in triggering the blast transformation. If more than one enzyme is involved in the triggering event, then how could it be possible to stop the whole process by inhibiting only one of these enzymes? To answer this question, one can imagine a series of pathways for the production of these enzymes as shown below.

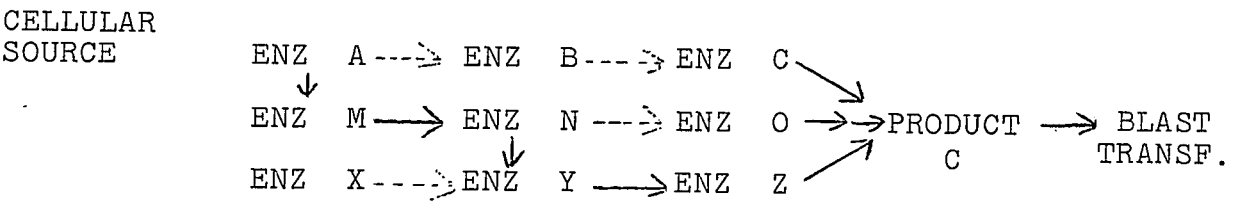
Pathway A (Series)



Pathway B (Parallel)



Pathway C (Cascade effect)



The blocking of any of these enzymes would result in preventing the lymphocyte blast transformation and would still agree perfectly with the data. Also, all the data reported in this work are compatible with the idea that more than one enzyme is involved. However, kinetic experiments using TLCK and TPCK failed to give any indications on the order in which these inhibited enzymes acted and therefore gave no indications as to which one of the three proposed pathways is most likely to occur.

WHERE DOES THIS ENZYME COME FROM?

The inhibitory action of the SBI and synthetic inhibitors did not indicate the possible sources of the enzyme(s).

1. The Plasma

The plasma, which is known to contain a multitude of enzymes, could certainly be one possible source. However, studies using different kinds of plasma or serum (autologous plasma, autologous serum, dialysed FCS, TLCK-treated FCS) showed similar degree of inhibition of the SBI in every case. Even if it (they) originated from the plasma, the enzymes would have certainly been inhibited by the irreversible binding of TLCK in experiments where FCS was pre-treated with TLCK before being added to the cultures. However, the cells cultured with TLCK treated FCS had a good response to mitogen and showed the same degree of inhibition in presence of the SBI as the cells cultured with autologous plasma. Thus the plasma, as a possible source of the hypothesized enzyme(s) was rejected.

2. The Macrophages and the Granulocytes

The macrophages and the granulocytes present in the cell culture could certainly be the source of the inhibited enzyme(s). Recently, numerous reports described

the role of the macrophages in lymphocyte blast transformation. Rosenstreich et al (19) report the absolute macrophage dependency of the T lymphocyte activation by mitogens and show, with the aid of Marbrook chambers, that components released from the macrophages are essential to the response of T lymphocytes to mitogens. As previously mentioned in the Introduction, macrophages contain a multitude of lysosomes filled with proteases that could easily be released to the medium. Havemann and Schmidt (73) have reported that the supernatant of adherent cells in antigen stimulated cultures caused an enhancement of the stimulation by 2 to 3 times and was shown to contain at least two proteolytic enzymes. Vischer and his collaborators recently reported that azurophil granules, present in polymorphonuclear leukocytes, contained two neutral proteinases, an elastase and a chymotrypsin like Cathepsin G, and showed that these two enzymes stimulated human peripheral blood lymphocytes and mouse spleen cells but not thymic cells from either. They also indicated, with the aid of protease inhibitors, that lymphocyte stimulation was dependent on the proteolytic activity of the enzymes (33). In view of this last report, it is very hard to reject the possibility that macrophages and granulocytes are the source of the hypothesized enzyme.

However, all our experiments designed to show the involvement of the macrophages and the granulocytes in the production of the enzyme(s) were inconclusive.

3. The Lymphocytes

Other reports suggest that the lymphocytes themselves could be the source of the hypothesized enzyme(s). Indeed Tokes (74) recently reported that there is a protease activity associated with the lymphocyte surface. Grazel et al (72) reported that at pH 7.5, lymphocytes had some proteolytic activity both at their surface and in the crude extract. It is also noteworthy that Weissman and his collaborators (75) and Havemann et al (73) reported that stimulation of lymphocytes by mitogens caused a release of protease activity.

The suggestion that lymphocytes are the possible source of the proteolytic activity becomes very attractive, especially since it has been shown in this laboratory by my colleague, P. Mazodier, that lymphocytes pre-treated with TLCK are still able to stimulate but lose their capacity to respond in the MLR. This would indicate that TLCK inhibits blast transformation by acting on the lymphocyte. If the macrophages were the source of the hypothesized enzyme(s), pre-treating only one of the two different populations would result in leaving one

population perfectly intact and able to produce the enzyme(s) and therefore no inhibition would be apparent.

The fact that the mitogenic response of the lymphocyte is enhanced by cell crowding would also favor the hypothesis of a membrane bound protease(s).

Therefore, the origin of this enzyme activity is still ambiguous. It could be an endogenous lymphocyte enzyme, as predicted by the hypothesis, or it could be coming from other cells present in the culture. All the data are in accordance with both suggestions (i.e. macrophage or lymphocyte).

SITE OF ACTION OF THE HYPOTHESIZED ENSYME(S)

If the source of the proteolytic activity is still not very well defined, its site of action is almost certain. Hirschhorn et al (70) interpreted the inhibitory effect of EACA by indicating that the agent acted by inhibiting some essential protease step at the level of the cell nucleus. Following this interpretation, many authors have reported data leading to the conclusion that the action of the protease might be at the nucleus. Darzynkiewicz et al suggested that TPCK and TLCK inhibited proteases present in the nuclear chromatin. Weissmann and co-workers (75) have also suggested that proteolysis of histones might be an essential early step in the

lymphocyte transformation and that it could be inhibited by TLCK. Saito et al (71) seem to agree with the inhibition of lymphocyte blast transformation being due to the inhibition of intracellular proteases. All these authors had one thing in common: they were using synthetic protease inhibitors.

Even though it was unlikely that SBI (M.W. 24,000) penetrated into the cell, in order to eliminate the possibility that the "essential proteolytic event" took place inside the cell, SBI was covalently bound to Sepharose beads and added to the culture of lymphocytes. Since the bound SBI still caused an inhibition of both Con A and MLR response, this possibility was then rejected.

The proteolytic step inhibited by SBI and probably by the synthetic inhibitors is, then, a surface event and once again fits perfectly with Kaplan and Bona's hypothesis. Vischer et al, in their recent paper (33), although they do not propose any specific site of action, now seem to agree that an essential proteolytic step occurs at the membrane level. What then, could be the side of action of this (these) ensyme(s) on the membrane?

A few years ago, Kast published a descriptive article suggesting that lymphocyte transformation as well as initiation of malignant change involved the proteolytic

cleavage of a peptide from a membrane bound protein (78). He speculated that this specific peptide (that he calls the detendomer) was the trigger for cell transformation and maintains he has evidence that a peptide including the sequence Pro-Thr-Cys-Pro-Pro-Pro is the locus for the action of the proteolytic triggering event. This is an interesting suggestion and would certainly fit well with the hypothesis of Kaplan and Bona. Also, Hartmann and Bokisch showed that C3b (part of the complement system) could stimulate murine B cells (79). Hart and Streilein (31) have presented results that are consistent with this hypothesis and suggest that the necessary proteolytic step caused lymphocyte transformation by generating C3b from the cell-bound C3 or C3 being released from macrophages.

A large number of publications dealing with mouse cells tend to indicate that only B lymphocytes respond when proteases are added as mitogens. Indeed, Kaplan and Bona themselves (6), along with Vischer (5), Hart and Streilein (working with hamster cells) (31), and Vischer et al (33) report that only B cells are affected by small doses of proteases. One way to reconcile these observations with my results (dealing with mouse cells) showing an inhibition of the response to T lymphocyte

mitogens is to suppose the presence, in the T lymphocyte, of an endogenous enzyme, different enough from the added proteases and yet still inhibited by the protease inhibitors. The added protease would then be different enough that it could not mimic the action of the endogenous enzyme. Another hypothesis could suggest the presence of a sensitive membrane substrate (peptide or protein or detendomer) which would be exposed externally in the case of the B lymphocytes but not in the case of the T lymphocytes where only the endogenous enzyme could reach it.

As for the human cells, no problem arises since no one has shown that the added proteases act on one particular population.

DIRECT MEASUREMENT OF THE PROTEOLYTIC ACTIVITY

Before making my closing remarks, I would like to report that several experiments were done in order to measure directly this proteolytic activity essential to the blast transformation induced by any kind of agent. A series of experiments consisted in adding the culture medium of stimulated cells or even the stimulated lymphocytes themselves to a trypsin substrate and measuring any degradation caused by the hypothesized enzyme(s).

Because of the large amount of proteolytic activity present in the non-stimulated cell culture, probably due to the presence of plasma, this approach revealed no conclusive results. The second approach consisted in an antigen-antibody diffusion test. Medium in which human lymphocytes had been stimulated by different agents, was confronted by rabbit anti-bovine trypsin antibody. There was no precipitation line observed between the antibody and (1) the medium, (2) the intact cells and (3) the broken cells while the control bovine trypsin gave a sharp white precipitation line. The same results were obtained when the medium and the cell extract were concentrated 16 times by lyophilization. Therefore, all that we know about the proteolytic activity has been learned <u>indirectly</u> with the aid of protease inhibitors.

It may seem curious that trypsin (or a trypsin-like enzyme), an enzyme mostly found in the pancreas, is involved in the triggering of blastogenesis in lymphocytes. But trypsin is not only found to be involved in lymphocyte transformation; a few years ago Schnebli and Burger (80) showed that protease inhibitors restricted the growth of transformed cells. These authors presented strong evidence showing that an inhibitor-sensitive protease-like activity

was required by malignant transformed cells for unrestrained growth. They also showed that this proteolytic activity had the characteristics of trypsin or a trypsin-like enzyme acting at the membrane level.

A trypsin-like proteolytic step is also thought to be necessary for the triggering of the egg, another resting cell, to undergo rapid increase in cellular activity. Indeed, Epel and his collaborators (81) (82) have shown that upon fertilization, sea urchin eggs released some macromolecules which he calls "fertilization products (FP). These FP were found to contain protease activity which is inhibited by SBI.

A trypsin-like proteolytic event seems to be a common denominator whenever a resting cell is switched on and undergoes rapid increase in cellular activity. Lymphocyte transformation, malignant transformed cells and fertilization of an egg cell all have this in common: their state of very low cellular activity can be changed to a very active one and all show the involvement of a proteolytic step.

Although the role of protease inhibitors in lymphocyte transformation has been clearly established, a number of questions remain unresolved. Separation and

selective stimulation of T and B lymphocytes, before addition of protease inhibitors, would help to clarify whether one or the other is the specific target of the essential proteolytic step. Also, since Kaplan and Bona's work was done with trypsin, all experiments were aimed at the possible role of trypsin-like enzyme. In view of the recent findings of Vischer et al (33) demonstrating that a chymotrypsin-like enzyme is found in human lymphocytes and that this triggered blast transformation, some experiments (i.e. pre-treated plasma with TPCK instead of TLCK) should be repeated, using chymotrypsin inhibitors.

Nevertheless, there is no doubt that at least one proteolytic step, acting at the membrane level and inhibited by SBI, is involved in the blast transformation of human and mouse lymphocytes as predicted by Kaplan and Bona's hypothesis. Whether this proteolytic activity comes from the lymphocytes or from another source and whether T or B human lymphocytes are affected by these proteases remains to be solved.

I think these remain two very interesting questions and I am sure they can trigger the interest of numerous scientists.

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