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El Simposio «Lo bueno y lo malo de las semillas de leguminosas» se realizó el 26 de Octubre de 1994 en las instalaciones de la Asociación Cultural Humboldt, Caracas, Venezuela.

The Symposium «Legume seeds friends and foes» was held at the Asociación Cultural Humboldt in Caracas on the 26 of October of 1994.

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La edición del presente volumen, donde se recogen las ponencias presentadas en el Simposio «Lo bueno y lo malo de las semillas de leguminosas», fue realizada por el Dr. Andrés Carmona

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Legume seeds: Friends and foes

Andrés Carmona

Last October Dr. Werner G. Jaffé turned eighty years old. To pay homage to this leading figure of the Venezuelan scientific community, a symposium, entitled «Legume seeds: Friends and foes», was organized with the participation of prominent guest speakers and members of the research group founded by Dr. Jaffé at the College of Sciences of the Universidad Central de Venezuela (UCV), who covered different issues pertaining to legume seeds, from the antinutritional factors that they contain, to their genetic, agronomic and nutritive potential.

The symposium was opened with a personal account by Werner G. Jaffé regarding how he started his pioneer work on antinutritional factors in legume seeds. In a retrospective assay on the history of the Biochemistry and Nutrition Group, Abraham Levy-Benshimol portrayed the most relevant accomplishments of Dr. Jaffé's Group in relation to the identification, detection and characterization of protease inhibitors and lectins responsible in part for the toxicity of raw pulses. The scope of this presentation was widened to include recent studies on the effect of bean tannins and complex carbohydrates on the digestive process.

Four presentations were dedicated to the Classical Antinutritional Factors: Lectins and protease inhibitors. Arpad Pusztai and Susan Bardocz, from the Rowett Research Institute of Aberdeen, Scotland, presented detailed studies on the characteristics and consequences of lectin-intestinal mucosa interactions and the effect of phytohaemagglutinin (PHA) on intestinal cell proliferation. The specific recognition by lectins of receptors on the mucosal cells interferes with the metabolism of these cells, impairs their digestive/absorptive activities and promotes cell proliferation and turnover. Lectins such as PHA from *Phaseolus vulgaris* induce time dependent and reversible hyperplastic and hypertrophic responses which require a large supply of polyamines (putrescine, spermidine and spermine) from systemic blood. The findings of these studies suggest that lectins may limit tumor proliferation by channelling the available polyamines towards normal cells which are reversibly stimulated to proliferate. Therefore, if used judiciously, lectins may be employed to improve the physiological performance and bacterial ecology of the intestine. Subsequently, Dinah S. Seidl (UCV) spoke of the molecular characteristics of inhibitor-protease interactions and the isolation and purification of six trypsin-chymotrypsin isoinhibitors from *Phaseolus vulgaris* seeds including the interaction of these molecular species with the trypsins from various animals and man, and the results of a bioassay performed with adult rice weevils in which a inhibitor-rich fraction was added to the pea flour fed to the insects. In her overview on protein proteinase inhibitors, Yehudith Birk, from the Hebrew University of Jerusalem, addressed issues pertaining to the potency and specificity of protease inhibitors and their effects (growth depression and pancreatic hyperplasia) when fed to various animal species. Mention was made of the possible antitumorigenic effect of proteinase inhibitors, in which case these proteins may be considered beneficial factors contributing, therefore, to the nutritive value of legume seeds.

The next two presentations were dedicated to the Emerging Antinutritional Factors. Juscelino Tovar (UCV) spoke about the bioavailability of carbohydrates in legumes. The digestible starch fraction is slowly degraded by the pancreatic-intestinal enzyme system and promotes middling post-prandial glycemic and insulinemic responses. In contrast, at least 10% of the seed starch, comprised mostly by retrograded amylose, escapes digestion in the small intestine and is a substrate for microbial fermentation in the large bowel, generating volatile fatty acids which influence the colonic mucosa and the metabolism of various organs such as the liver. Andrés Carmona (UCV) addressed problems related with the extraction, purification and chemical characterization of bean polyphenols and the *in vitro* interactions of the tannin fraction with digestive enzymes and other proteins. After extraction with 1% HCl in methanol, the polyphenol fraction from black beans was shown to contain a large proportion of condensed tannins which formed both soluble and insoluble complexes with proteins. Several digestive enzymes, such as the pancreatic proteases and amylase and the brush border disaccharidases, were strongly inhibited by these thermostable pigments. The use of a variant of the vanillin reaction in glacial acetic acid was suggested to

overcome some shortcomings of the traditional procedure in methanol, widely recommended for tannin analysis in legume seeds.

In the final section of the symposium three papers were presented which focused on the agronomic potential and genetic improvement of legume crops and on the effect of processing on antinutritional factors in legumes. Juan de Jesús Montilla and Julio Viera from the Colleges of Veterinary Medicine and Agriculture of the Universidad Central de Venezuela, respectively, outlined several features of legume crops from their ability to form symbiotic associations with *Rhizobium* bacteria and the consequences of such interaction to nitrogen fixation and soil improvement to their reproductive behavior and described a program of genetic improvement able to produce populations with a high frequency of desirable phenotypes. In the case of *Canavalia ensiformis* it has been possible to domesticate this species as indicated by increases in plant size and germination potential of the seeds and a decrease in the plant dependency on ambient humidity. These changes have been accomplished without affecting the yield, under experimental field conditions. The Symposium was closed by Irvin E. Liener who recounted his contributions, which expand from 1946 up to now, leading to the identification of the factors which contribute to the poor nutritive value of raw soybeans. After removal of the lectin or the protease inhibitor activities, it was concluded that the former was responsible by 50% of the inhibition of growth in rats while the latter accounted for another 40%, of which two-thirds could be attributed to the Kunitz trypsin inhibitor and one-third to the Bowman-Birk trypsin-chymotrypsin inhibitor. The remaining 10% was attributed to the intrinsic low digestibility of raw soy proteins. The increase in nutritive value of heat-treated soy meals depends on both the extent to which the lectin and protease inhibitors are inactivated by heating and upon the influence of other factors such as temperature, duration of heating, particle size and moisture conditions. In any case, excessive heating should be avoided to prevent damage to the nutritive value of the protein. Therefore, the appropriate duration and intensity of the treatment should result from a compromise between inactivation of heat-labile antinutritional factors and the decrease in nutritive potential of the resulting product.

The following contributions reflect what happened along the symposium. It is certainly pleasant that they are being published by Archivos Latinoamericanos de Nutrición, a Journal whose first Editor was Werner G. Jaffé.

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Como llegué al estudio de las leguminosas

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RESUMEN. El autor presenta, en este artículo, un breve recuento personal de como se involucró en el estudio de las leguminosas desde una perspectiva nutricional y toxicológica. Luego de observar que la dieta de los campesinos venezolanos incluía con frecuencia caraotas (frijoles) negras cocidas y un pan de maíz llamado arepa, realizó experimentos nutricionales que lo llevaron a reconocer que las semillas crudas de leguminosas contenían factores antinutricionales de naturaleza termolábil y que las proteínas de estos granos se complementaban, nutricionalmente, con las del maíz. Entre los factores antinutricionales aisló una fracción hemaglutinante que luego fue extensamente caracterizada. Basado en las propiedades hemaglutinantes y tóxicas de las lectinas logró reconocer, en las semillas de *Phaseolus vulgaris*, la existencia de cuatro tipos diferentes. Su trabajo con las dietas a base de semillas de leguminosas le llevó a participar en la búsqueda de un factor de crecimiento que luego fue denominado vitamina B_{12}.

SUMMARY. How did I begin nutritional studies in legume seeds?. In this paper the author presents a brief account of his involvement in the study of legume seeds form a nutritional and toxicological perspective. After observing that the Venezuelan peasants ate diets which often included cooked black beans and a form of corn bread called arepas, he performed nutritional trials which led him to recognize that raw beans contained thermolabile antinutritional factors and that their proteins were complementary to those of corn. Among the antinutritional factors, he isolated a hemagglutinating fraction which later was further characterized. Based on their properties he recognized the existence of four different types of *Phaseolus vulgaris* cultivars. Research on the nutritive value of bean diets also got him involved in the identification of a growth factor later called vitamin B_{12}.

Quizás fue el recuerdo del hambre que pasé de niño, a consecuencia de la situación de escasez que atravesó mi país natal en los años posteriores a la Primera Guerra Mundial, lo que me impulsó al estudio de la nutrición humana. Todavía recuerdo, perfectamente, la distribución equitativa de las raciones de comida entre los hermanos en la mesa familiar y el sentido de frustración cuando éstas resultaban muy pequeñas. Igualmente recuerdo las caravanas de soldados que regresaban del cautiverio y el triste aspecto que tenían. Estas tempranas impresiones causaron en mi un profundo sentimiento antibélico y una continuada preocupación por el hambre ajeno y por ello, por la Ciencia de la Nutrición y sus aspectos sociales.

La selección de uno de los temas que me ha ocupado en mi labor de investigación, las leguminosas, también está relacionada con eventos bélicos, esta vez con la Segunda Guerra Mundial. En el curso de esta conflagración, las tropas japonesas ocuparon las Islas de Indonesia, entonces colonia holandesa. Con esta acción se recortó bruscamente la oferta mundial de quinina, producida exclusivamente en esta zona. En aquel entonces, los remedios antipalúdicos sintéticos no estaban disponibles. Yo trabajaba en esos años para un laboratorio farmacéutico particular, cuyo producto más importante era un jarabe de quinina, muy popular para el combate de la malaria tan extendida en Venezuela.

Sabiendo que la quinina se obtenía de la corteza del árbol de quina, el cual se encontraba en la zona andina de Suramérica, resolví, junto con un amigo botánico, indagar sobre la posible presencia de esta especie botánica en los bosques de los alrededores de Caracas. En estas excursiones teníamos que pernoctar en las pequeñas casas de los campesinos de la zona, y se nos ofrecían comidas que, invariablemente, consistían de arepas de maíz y de caraotas (frijoles) negras, a veces con algún aderezo.

Curioso por saber cómo una dieta tan monótona y sencilla podía cubrir los requerimientos fisiológicos, empecé a realizar experimentos con ratas blancas que se tenían para pruebas toxicológicas en la empresa donde trabajaba.

Estos ensayos condujeron a cuatro observaciones de interés, y cuya profundización y explotación determinaron en buena medida mi labor de investigación durante los siguientes treinta años. Las observaciones fueron las siguientes:

1. las ratas que recibieron una dieta de caraotas negras y maíz molido, siempre reforzada con vitaminas y minerales, murieron después de, aproximadamente, dos semanas, con síntomas de diarrea.

2. la sustitución de las caraotas crudas por otras sometidas a cocción previa, secado y molienda condujo a un crecimiento satisfactorio de los animales.

3. la administración de dietas a base de cualquiera de los dos componentes (maíz o caraotas cocidas) no promovió el aumento de peso de las ratas.

4. los animales que recibieron la dieta de maíz y caraotas cocidas crecieron satisfactoriamente y se reprodujeron. Sin embargo, en la segunda generación se observó retardo del crecimiento y alta mortalidad de las crías. Estos síntomas se corrigieron cuando se le suministró a las madres un extracto hepático.

La incapacidad del maíz o las caraotas cocidas para satisfacer, por separado, los requerimientos alimenticios de las ratas y el elevado valor nutritivo de la mezcla, señaló el efecto complementario entre ambos componentes y abrió el camino para el estudio de los aminoácidos esenciales limitantes de sus respectivas proteínas.

El efecto beneficioso del extracto hepático sobre la capacidad reproductiva de las ratas alimentadas por largo tiempo con la dieta reforzada con todas las vitaminas conocidas hasta la fecha, se interpretó como indicio de la existencia en dicho extracto de un factor o factores de crecimiento esenciales y todavía desconocidos, cuya búsqueda nos ocupó por muchos años.

Preparamos concentrados del extracto hepático y se estudiaron sus efectos biológicos en ratas y lactobacilos. Estos se compararon con los de una fracción parcialmente purificada del factor antianémico de Castle, que nos fue suministrada por el Profesor Karrer de Zurich. Por sus características similares, se sospechó que se trataba del mismo factor. Como es conocido, en 1948 un grupo de investigadores norteamericanos logró su cristalización y se le denominó Vitamina B_{12}.

La alta mortalidad de las ratas que consumían las caraotas crudas, indicaba la presencia en ellas de uno o varios factores tóxicos termolábiles. En trabajos posteriores se logró aislar uno de ellos, el cual presentó actividad hemaglutinante. Una vez purificado se le determinó su peso molecular, su carácter de glicoproteína y su adhesión a hematies y a tejido intestinal. Una comparación de las actividades tóxicas y hemaglutinantes de semillas provenientes de varios cultivares de *Phaseolus vulgaris*, permitió establecer la existencia de cuatro diferentes tipos de lectinas en dichas semillas, las cuales se diferencian por su especificidad frente a eritrocitos de varias especies animales, a su toxicidad en animales cuando se les suministraba por vía intraperitoneal o sub-cutánea, a su actividad mitogénica frente a linfocitos humanos, a su contenido de carbohidratos y a su termolabilidad.

De los cuatro tipos de lectinas que se encontraron en diferentes variedades de semillas de *P. vulgaris*, sólo dos demostraron poseer una fuerte actividad tóxica, la cual resultó relativamente resistente a la cocción. El calentamiento por dos horas a 92 °C, la temperatura a la cual hierve el agua en la Ciudad de México, no eliminó por completo la toxicidad. En cruces realizados entre un cultivar de alta y otro de baja toxicidad, se pudo observar que el carácter del primero se hereda como un factor dominante.

La especificidad de la acción hemaglutinante frente a los eritrocitos de diferentes especies resultó muy útil para distinguir entre diversos cultivares de esta leguminosa y nos sirvió, no sólo, para investigar la homogeneidad de lotes de semillas, porque dicha prueba se puede efectuar con una sola semilla, sino también para demostrar que las llamadas lectinas leucoaglutinantes, consideradas como no eritroaglutinantes debido a su poca actividad frente a eritrocitos de conejo, eran capaces de aglutinar glóbulos rojos de otras especies.

Para la fecha de mi llegada a Venezuela en 1940, no existía ninguna facultad de ciencias y mucho menos una cátedra de bioquímica. Se había contratado a un profesor catalán de fisiología, el Dr. Augusto Pi Suñer, para organizar el Instituto de Medicina Experimental, quien me invitó a ayudarle en esta tarea, pero no logró conseguir para mí un nombramiento remunerado como personal de la Universidad Central de Venezuela, la cual tenía, en esa época, un presupuesto de sólo catorce millones de bolívares. Por esta razón tuve que aceptar la oferta de la empresa farmacéutica antes mencionada. En su laboratorio de control me encontré con unas instalaciones básicas muy limitadas y una pequeña biblioteca con escaso material bibliográfico. Dos médicos asistían, por horas, como asesores para el desarrollo de nuevos productos. De los cinco años de mi actividad en esa empresa quedaron 24 publicaciones científicas.

Al ingresar a la carrera universitaria, tuve la oportunidad de trabajar con estudiantes tesistas, una actividad que siempre me ha complacido mucho, y que nos permitió profundizar en temas y problemas ya abordados antes, lo que expandió el horizonte de nuestro trabajo. Considero una gran suerte el haberme encontrado, desde entonces, con un grupo de alumnos excelentes. Sin ellos no hubiese logrado jamás una labor científica de alguna relevancia.

Desde que me inicié en el campo de las lectinas, éste ha adquirido evidentemente una amplitud e importancia que entonces apenas se podía vislumbrar. En un artículo de revisión que el Dr. I.E. Liener me comisionó para su libro «Toxic Constituents of Plant Foodstuffs» en 1969, me atreví a pronosticar que el estudio de las lectinas, probablemente, iba a experimentar un enorme desarrollo cuando se hubiera logrado describir, con mayor claridad, la reacción lectina-receptor y la ocurrencia de múltiples receptores en diversos tejidos y materiales biológicos.

El programa de este simposio, y del cursillo sobre lectinas programado conjuntamente, demuestra con claridad la multitud de fenómenos fisiológicos cuya descripción se logra gracias a este campo de investigación. Considero, por lo tanto, que fue un golpe de suerte que se me presentara la oportunidad de dedicarme, desde sus inicios, a este campo tan relevante en la actualidad.

Me emociona sobremanera encontrarme en este momento frente a distinguidos investigadores que han contribuido de manera fundamental en el avance de los conocimientos sobre la estructura, composición, modo de acción, efectos antinutricionales y tóxicos y posibles aplicaciones médicas de las lectinas y otras biomoléculas aisladas de semillas de leguminosas.

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The biochemistry and nutrition group: 30 years of research in a developing country

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SUMMARY. The most relevant results of 30 years of research from the Group of Biochemistry and Nutrition are presented. Research was focused mainly around the identification and detection of the heat-labile toxic factors present in legume seeds of human consumption, namely protease inhibitors and lectins with especial emphasis on their isolation, molecular characterization, mechanistic and nutritional relevance of both protein groups. The antinutritional effect of the polyphenols, thermolabile compounds present in colored seeds, has also been studied as well as the impact of seed complex carbohydrates on the digestive process.

RESUMEN. El grupo de bioquímica y Nutrición: 30 años de investigación en un país en desarrollo. Se presentaron los principales aportes del Grupo de Bioquímica y Nutrición en 30 años de investigación. Ésta se ha centrado en la identificación y detección de los factores tóxicos termolábiles presentes en las semillas de leguminosas de consumo humano, tales como los inhibidores de proteasas y lectinas, con énfasis en el aislamiento, caracterización molecular, mecanística y relevancia nutricional de ambos grupos de proteínas. También se han estudiado los polifenoles compuestos termolábiles presentes en las semillas coloreadas, como agentes antinutricionales y el impacto de los carbohidratos complejos de las semillas en el proceso digestivo.

INTRODUCTION

After having found that raw black beans were toxic, while the cooked ones constituted the basic diet of most of the underdeveloped peoples of Latin America. In the sixties our research concentrated mainly around the identification and detection of the heat labile toxic factors in legume seeds. They turned out to be the inhibitors of digestve enzymes and the lectins. These two groups of proteins, their isolation, molecular, mechanistic, and nutritional characterization occupied our research efforts during two more decades.

Technics used in early studies on the structure of the active sites of the Bowman-Birk inhibitor from soybeans and its interaction with trypsin and chymotrypsin in Dr. Irvin Liener's laboratory in the University of Minnesota, were applied by Dinah Seidl to the determination of the sites on the six isoinhibitors purified from black beans [1].

All were double-headed and interacted independently and simultaneously with trypsin and chymotrypsin, as shown by the electrophoretic migration pattern on cellulose acetate of their binary and ternary complexes with the enzymes. Their differences in electrophoretic migration were justified by their aminoacid composition, which were found to be between 51 for inhibitor IV and 83 for inhibitor I [1].

During specificity screening of black bean extracts an «odd» protein molecule was detected which inhibited microbial enzymes, such as subtilisin and proteinase K, but did not interact with either animal digestive or plant thiol enzymes [2]. The unusual characteristic of this inhibitor was its specific recognition of human leucocyte elastase. The so called «subtilisin inhibitors» (SI) were later found and purified, in addition to black beans, from broad beans, chick peas and Jack beans, (Table 1). Their molecular masses were around 9 KD and their isoelectric values between pH 4 and 6.3.

SI reacted with subtilisin by the «standard mechanism» proposed by Laskowski Jr. for the interaction of the trypsin inhibitor with trypsin [4]. In fact, SI molecules having split active sites were detected, and resynthesis of the bond was achieved [3].

Structural differences among the SI were detected mainly

in their stability under denaturing conditions and in immunochemical assays. Here, antibodies raised against Jack bean (*Canavalia ensiformis*) SI, which interacted with SI from other species and varieties of the Canavalia genus, did not recognize the purified inhibitors from either black beans, chick peas or broad beans [3].

TABLE 1
SUBTILISIN INHIBITORS CONTENT OF VARIOUS LEGUME SEEDS

Species	Subtilisin Inhibitors (mg/100 g dry seeds)
Black beans (*P. vulgaris*)	7
Chick peas (*C. arietinum*)	16
Jack beans (*C. ensiformis*)	21
Broad beans (*V. faba*)	28

Modified from reference 3

The nutritional implications of the bean trypsin inhibitors were focused on by studying the varying degrees of sensibility of the digestive enzymes of several animal species including man, toward these molecules, which are not necessarily inactivated during cooking.

Original methods to detect inhibitors in single seeds [5], and others to determine toxicity in microquantities were developed as part of our research [6].

Studying lectins in 1968 we demonstrated for the first time the leucoagglulinating and mitogenic properties of Concanavalin A [7]. This was followed by the observation of the toxic effect of this lectin on cultured cat kidney cells. This was one of the first reports of lectin toxicity on cultured cells [8].

At the same time we were interested in the inheritance of the factor or factors responsible for the haemagglutinating activity of Phaseolus vulgaris seeds. It was found that the genetic analysis pointed to a single dominant trait of inheritance of the plytohaemagglutinin (PHA) [9].

Jaffé, Brücher and Palozzo studying numerous genetically pure cultivars of *Phaseolus vulgaris* found that they could be classified into 4 groups according to their differences in haemagglutinating specificity, oral and intraperitoneal toxicity and mitogenicity. The simple haemagglutination test could then be used to predict toxicity in edible beans [10].

The lectins of each group were studied *in extenso* with Pedro Bonay. They were shown to be isolectins with similar but not identical biological properties [11,12]. Isolectins from the latex of *Hura crepitans* were also characterized and, interestingly, they are different from the one present in the seeds of the same plant [13].

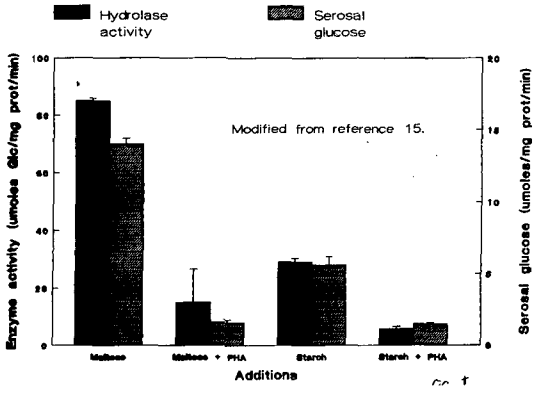
The effect of PHA on the absorption and transport of glucose in the small intestine was also studied. The lectin from a toxic variety reduced both absorption and transport of glucose [14]. Glucose metabolism as judged by lactate formation was not affected (Table 2). In other experiments, the inhibition of intestinal maltase and glycoamylase with a decrease of glucose absorption, was also demonstrated, Figure 1 [15].

TABLE 2
EFFECT OF PHA ON INTESTINAL GLUCOSE TRANSACTIONS

Lectin* µg/ml	Glucose Absorption	Glucose Transport	Lactante Production
	µmoles/5cm/20 min		
0	26.4 ± 2.2	2.9 ± 0.2^{a}	13.3 ± 0.5^{a}
50	19.3 ± 2.2^{a}	1.4 ± 0.2^{a}	12.9 ± 0.5^{a}
100	16.1 ± 1.6^{a}	0.8 ± 0.2^{ab}	12.5 ± 0.7^{b}
200	15.2 ± 1.5^{ab}	0.7 ± 0.2^{a}	12.1 ± 0.6^{b}

* Pre-incubation time: 15 minutes
Means sharing the same superscript are not statiscally different ($p<0.05$)
Modified from reference 14

FIGURE 1
Effect of PHA on brush border maltase and glycoamylase



The effect of some plant polysaccharides on lectin activity was investigated [16]. Arabic gum enhaces lectin haemagglutinability. The more glycosilated the lectin, the greater the stimulatory effect of the gum. The unglycosilated PHA retains the same agglutinating activity as the native, glycosilated lectin. However, the gum has no effect on the modified lectin, suggesting that the interaction between gum and lectin is of a carbohydrate-carbohydrate nature.

The activity of pancreatic α-amylase is inhibited by PHA. No such effect was observed with the unglycosilated lectin, suggesting again the importance of the sugar moiety in the biological properties of lectins [17].

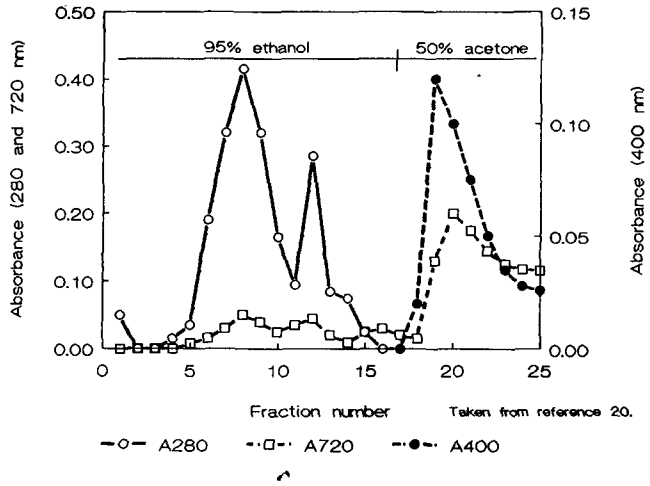
In addition, although heat treatment (90 min, 96 °C) inactivates PHA hemagglutinating activity, a remaining anti amylolytic effect is still observed (Figure 3). This finding stresses the importance of thermoresistance of some legume antinutritional factors.

In 1973 Jaffé reported that the digestibility of colored cooked beans was lower than that of white seeds, and suggested that this difference could be attributed to the larger polyphenol content of the red and black beans. Later on, works from the Instituto Nacional de Nutrición de Venezuela, revealed that black bean broth impaired the weight gain of animals fed casein [19].

At that time Andrés Carmona joined our Research Group and started a new line aimed to characterize the black bean polyphenols, selecting the best conditions to extract, purify and estimate them. The antinutritional effect of purified bean tannins was also evaluated.

The first outcome was the clear separation of bean tannins by adsorption chromatography resulting in an elution pattern similar to that reported for sorghum grain, Figure 2 [20]. The in vitro inhibitory α-amylase activity of the condensed tannins was not eliminated by autoclaving, however it was abolished by the polyphenol complexing agent polyvinyl polypyrrolidone [20].

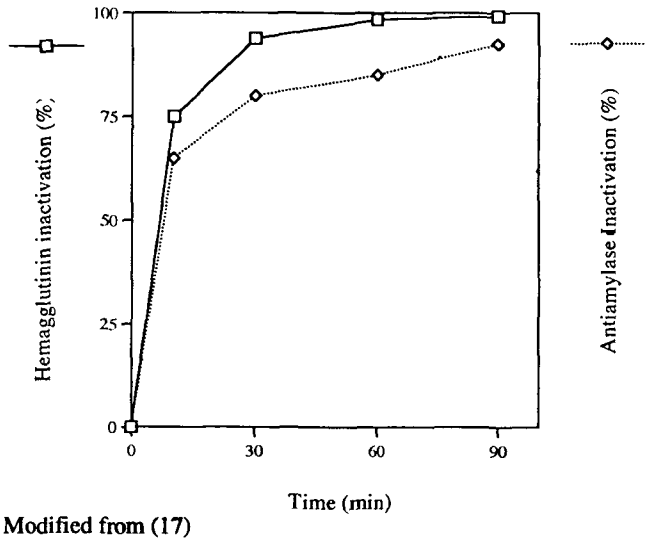
FIGURE 2
Adsorption chromatography of black bean polyphenols



A great deal of work has bean put by our Research Group into the determinion of the specificity and selectivity of the most commonly used spectrophotometric assay procedures for tannin estimation. Those based on reductive power (Prussian Blue and Folin-Denis) did not distinguish between tannins and non-tannin polyphenols. This could be accomplished by using the vanillin raction if corrected by substracting the colour contributed by the seed extracts [21,22].

Recently some of the shortcomings of the traditional vanillin reagent, such as the interference from non-tannin polyphenols present in the raw bean extracts, have been overcome by using acetic acid as solvent. This procedure increases sensitivity and decreases background noise [23].

FIGURE 3
Effect of heat treatment on hemagglutinating and antiamylase activities of PHA



Modified from (17)

The incorporation of Gina Borges in 1991 allowed us to explore in detail the interaction of tannins with several components of the digestive machinery, both in vivo and in vitro. Apparently, the inhibition of pancreatic and brush border carbohydrate hydrolases is stronger than that of glucose absorption.

From the pioneering work of David Jenkins it is known that legume seeds elicit a low glycemic response in humans [24]. This prompted us to go into a new research field, now headed by Juscelino Tovar.

It was demonstrated that in legume seeds the indigestible residue represents a larger fraction than that chemically estimated. Therefore, enzymatic methods allow for a more precise analytical calculation of the fiber content [25]. These observations must be taken into account when the physiological implications of legume intake are to be considered.

Lately it was found that the indigestible residue of black beans (*P. vulgaris*) inhibits the in vitro activity of trypsin and pancreatic α-amylase [26]. The starch limited hydrolysis could account in part for the low glycemic response.

It is also of interest that thermic processing of pulses produces indigestible fractions in greater proportion than in other amylaceous food stuffs [17]. These changes decrease polysaccharide biodisponibility.

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Characteristics and consequences of interactions of lectins with the intestinal mucosa

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SUMMARY. Lectins are essential and omnipresent plant (glyco)protein constituents and are ingested daily in appreciable amounts by both humans and animals. As they are biologically highly active, their consumption may have serious consequences for metabolism and health. Lectins, by virtue of their stability and specific recognition and binding by gut brush border epithelial cells, are potent exogenous metabolic growth signals for the gut and the body. As a result of their binding to surface glycans they may affect the turnover and loss of epithelial cells, damage the luminal membranes of the epithelium, interfere with their digestive/absorptive activities, stimulate shifts in the bacterial flora and modulate the immune state of the digestive tract. When eaten in relatively large quantities, these lectins have appreciable antinutritive effects for the consumers. In contrast, lectins which are not bound by the mucosa usually induce little or no harmful effects. From recent studies it is now realized that in addition to the major and sometimes dramatic effects of lectins on the gut which are mediated through their binding to pre-existing membrane glycosyl groups, lectins as metabolic signals, can also radically alter the state of glycosylation of the gut epithelium and thus further amplify their potent physiological effects. Accordingly, with the judicious use of dietary lectins it is now possible 'to engineer' the digestive tract for improved physiological performance and bacterial ecology.

RESUMEN. Características y consecuencias de la interacción de lectinas con la mucosa intestinal. Las lectinas de plantas son componentes esenciales y onmipresentes de naturaleza (glico)proteica que se ingieren en cantidades apreciables diariamente, tanto por humanos como por otras especies animales. Debido a su intensa actividad biológica intrínseca, el consumo de estas proteínas puede acarrear serias consecuencias, tanto a nivel metabólico, como de la salud en general. En virtud de su estabilidad y de su enlazamiento especifico por células epiteliales del tracto digestivo, las lectinas pueden actuar como potentes señales de crecimiento a nivel intestinal y del organismo en general. A consecuencia de su unión a glicanos de superficie, las lectinas pueden afectar los procesos de recambio y la descamación de las células epiteliales, dañar las membranas luminales del epitelio, interferir con los procesos de digestión y absorción, promover cambios de la flora bacteriana y modular el estado inmune del tracto digestivo. La ingesta de estas lectinas en altas cantidades produce efectos antinutricionales apreciables en los consumidores. En contraste, las lectinas que no se unen a la mucosa usualmente causan poco o ningún efecto deletéreo. Estudios recientes nos han llevado al convencimiento de que, además del efecto principal y a veces dramático de las lectinas sobre el intestino y el cual es mediado por su unión a grupos glicosilo pre-existentes en la membrana, las lectinas como señales metabólicas pueden alterar radicalmente el estado de glicosilación del epitelio intestinal y, por ende, amplificar su efecto fisiológico potencial. En consecuencia, con el uso ponderado de las lectinas de origen dietario sería posible «modificar a voluntad» el tracto digestivo para potenciar su respuesta fisiológica y optimizar la ecología bacteriana.

INTRODUCTION

Lectins, or more generally, carbohydrate-binding (glyco)proteins, are essential and omnipresent constituents of plants. As a major part of our food is of plant origin, appreciable amounts of lectins are ingested every day with potentially important consequences. Although some lectins are justly regarded as antinutrients because of their damaging effects on the gut and body metabolism, not all lectins are nutritionally toxic. During evolution man must have eliminated toxic plants from the diet, including those with highly toxic lectin constituents and/or learnt how to de-toxify others. Selection by agricultural means was also extensively exercised in favour of plant varieties which had low residual toxic components; for example the lectin content of some cultivated species of Alliaceae is much lower than in wild varieties. Moreover, plants have occasionally been selected because some of their constituents, including the lectins, possess properties beneficial for health (e.g. garlic).

It is clear that the process of selection by man of nutritionally high-quality crop plants has in the past been based on empirical observations. Although this can now be approached by rational and objective experimentation, direct experimental evidence for the involvement of individual food components in the regulation of gut metabolism is generally scanty. Thus, despite the commonly accepted view that the diet has a major effect on health, rigorous criteria for selection are still not readily available. However, because of the pioneering nutritional studies of Werner Jaffé and Irvin E. Liener, plant lectins may provide one of the few exceptions in this respect. Hopefully, from the sometimes dramatic interactions between ingested lectins and the intestinal mucosa and their consequences for gut and body metabolism, it may be possible in future to select and/or genetically engineer plants for their positive contribution to human and animal health.

Many lectins, regardless of whether they are from plants or bacteria, are potent exogenous growth signals and some can also mimic the action of metabolic hormones (1). Their biological activity is a direct consequence of lectin function: through recognition and binding to specific carbohydrates of receptors on surface membranes, they send signals and deliver messages to cells. Since interaction between lectins and cells depends on their specific recognition of membrane glycans, differences in the potency to bind to cells are mainly due to differences in the state of glycosylation of the cell membrane.

One of the main reasons for the extraordinary effectiveness of ingested lectins on the digestive system is that all lectins studied to date are highly resistant to proteolytic breakdown during gut passage (2). This, coupled with their specific and high reactivity with and binding to surface glycosyl residues of the gut epithelium, can induce major changes in the metabolism, turnover and loss of epithelial cells, may damage their luminal membrane, influence their absorptive capacity for both small and large molecules, stimulate shifts in the bacterial flora and interfere with the immune state of the digestive tract. It is now clear that lectins which avidly bind to the brush border are powerful metabolic signals and growth factors for the entire gut, particularly the small intestine, and induce fully reversible, dose- and polyamine-dependent hyperplastic growth. Other lectins for which no suitable glycosyl receptors exist on the villus surface are usually without any effect (2). These pass through the gut and are excreted in the faeces. However, some of these lectins may also reach the luminal surface of crypt cells whose glycosylation is different from that of fully mature enterocytes. If this is compatible with the sugar specificity of the lectin, binding will occur and anti-mitogenic effects may ensue, leading to a reduction in the rate of crypt cell proliferation and a general slowing down of gut metabolism. Accordingly, strongly mitogenic lectins are usually regarded as antinutrients because these can damage the gut at high doses and on continuous exposure, these lectins may possibly act as adjuvants of chemical carcinogens. Despite these negative attributes, mitogenic lectins can also have some beneficial effects because, at low, non-toxic doses, they can be used to stimulate growth in intestinal hypoplasia caused by parenteral feeding, gut resection and other gut lesions. However, anti-mitogenic lectins probably will have more numerous applications because, by reducing the rate of intestinal cell proliferation, they may also slow down the development of tumours of the large bowel. As most of these lectins are specific for mannose, they can serve as excellent and safe blockers of infections by type-1, mannose-sensitive, fimbriated pathogenic bacteria (3).

One of the best-known and most intensively studied dietary lectin is phytohaemagglutinin, PHA, from kidney bean (*Phaseolus vulgaris*). This is a powerful exogenous metabolic signal and growth factor for the gut (for references see 1). Because of its stability and biological activity of avid binding to receptors of endogenous growth factors, hormones and bacteria expressed on the luminal brush border membrane, PHA delivers potent messages to the epithelial cells leading to changes in gene expression and cellular metabolism. Although it is not clear whether these are direct lectin effects or mediated through endogenous growth factors, there is definite evidence for the binding of lectins to cells of jejunal crypts (2). Therefore it is possible that the PHA signal may directly stimulate crypt cell proliferation and that all its dramatic effects on the gut are a consequence of this. As the state of glycosylation in the small intestine is intricately linked to the development and metabolism of its epithelial cells, major changes in the glycosylation of brush border membrane and cytoplasmic glycoconjugates can be expected as a consequence of the powerful physiological growth factor activity of PHA.

In contrast to the dramatic effects of strongly mitogenic lectins on gut metabolism and receptor glycosylation, the effects of non-mitogenic or poorly mitogenic lectins appear to be more subtle. Thus, the lectin of snowdrop (*Galanthus nivalis*) bulbs, GNA a strictly mannose-specific lectin is a particularly interesting example because although it binds to the crypt (2), its effect appears to be the opposite of PHA. By slowing down the rate of crypt cell proliferation, GNA induced a significant decrease in the length and cell numbers of the crypts and therefore its effects on brush border glycosylation were expected to be particularly revealing in comparison with that of PHA or other similar mitogenic lectins.

There is also convincing experimental evidence that, after binding, some lectins are endocytosed by the gut epithelial cells and there they may interact with internal glycan receptors. As a consequence of this additional metabolic signalling, the powerful physiological effects of PHA and other strongly mitogenic lectins are further amplified and extended, leading to increased rates of crypt cell proliferation, speeding up of epithelial turnover and major changes in the glycosylation of membrane glycans (1).

The most important conclusion emerging from recent studies of the effects of dietary lectins on the alimentary tract of higher animals is that the state of glycosylation of membrane

receptors of gut epithelial cells is highly influenced and in some instances dramatically changed by the presence of a number of biologically active components, particularly lectins, occurring in food/feeds (4). As glycosylation is of paramount importance in deciding how effectively growth factors, hormones, lectins and bacteria can bind to these receptors, the entire metabolic state, digestive/absorptive functions, bacterial ecology and health of the gut can be modulated by the diet through its lectin content.

In a recent histochemical study, by using digoxigenin-labelled lectins as histochemical reagents, comprehensive information on the carbohydrate structure of gut surface receptors was obtained, enabling us to define a low resolution membrane glycosylation map of the small intestine of rats exposed to different dietary lectins in the presence or absence gut flora (4). Although the resolution of digoxigenin-lectin staining at the light microscope level was low and fine structural features of epithelial cells could not be unequivocally located, the results gave an overall view of the state of glycosylation in the lumen of the small intestine. The staining clearly illustrated the presence of saccharide structures on the gut wall or in the cytoplasm of the epithelial cells which were available for possible interactions with dietary factors or resident or infecting bacteria.

In general the results confirmed and extended some of the commonly held views on the state of glycosylation of small intestinal brush border membranes and cytoplasmic glycoconjugates of both villi and crypts in weaned rats possessing a conventional microflora and well-fed on high-quality control diets (5-7). Thus, the villous brush border membrane contained mainly complex glycosyl structures and the expression of terminal mannosyl residues in it, characteristic for immature cells, was vanishingly small. Some of the membrane glycoconjugates were also fucosylated and/or α-2,3 sialylated (7). Although α-2,6 sialylation was absent on the brush border membrane or in goblet cells, these saccharide structures were abundant in the cytoplasm.

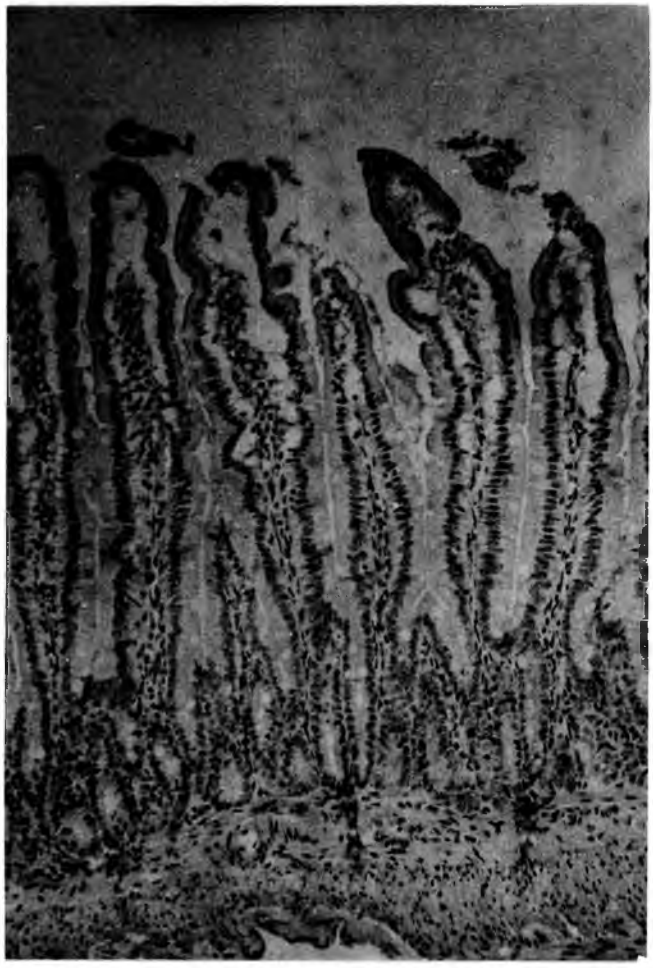
The main and most important finding of this recent study corroborated previous observations and showed conclusively that the state of glycosylation of both membrane and cytoplasmic glycoconjugates was modified by feeding rats on diets containing lectins. Some of these changes were also dependent on the presence or absence of bacteria in the lumen of the gut and, indeed, bacteria themselves could modify the glycosylation of luminal receptors (4). Although the differences between germ-free rats and those possessing a conventional microflora were mainly quantitative, they were obvious in some instances even with the qualitative histological techniques used and in general, the changes in glycosylation caused by lectins were more substantial than those by bacteria.

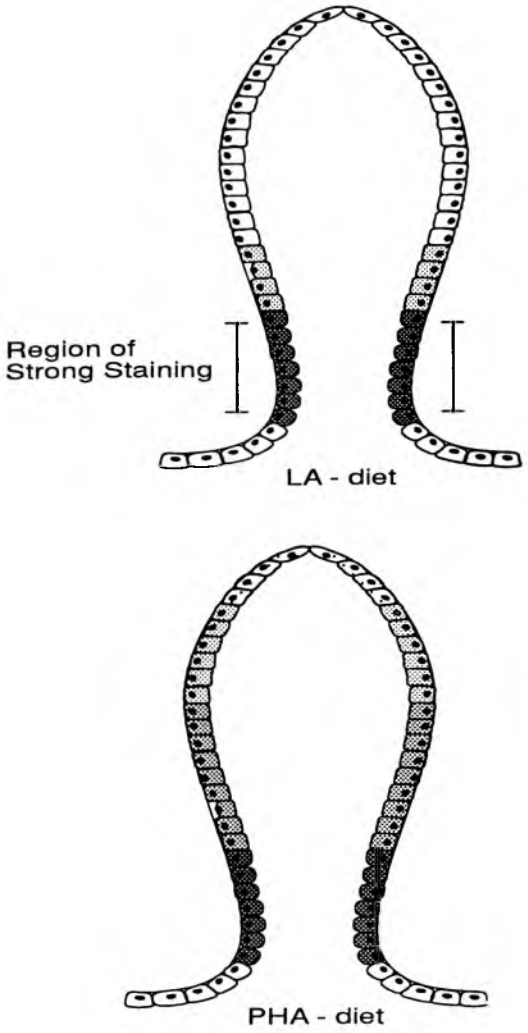
In conclusion, continuous oral exposure of rats to lectins induced major changes in the expression of both membrane and cytoplasmic glycoconjugates altering the structure of terminal saccharides. Some of these were appreciably influenced by the presence or absence of bacteria in the gut lumen. Moreover, some changes in glycosylation also led to major shifts in the bacterial flora of the small intestine such as were found with the dramatic overgrowth of *E. coli* in its lumen after feeding rats on PHA-diets and the successful blocking of this by the dietary administration of GNA (3). However, some of the physiological consequences of the changes induced by lectin exposure are not apparent at present. In general the shifts in glycosylation fall into one of the following categories:

a. *Changes in glycosylation by displacing endogenous ligands.*

Although in healthy rats the brush border membrane of the small intestinal epithelium contains mostly complex glycosyl residues and only few mannosyl terminals, most of these are not shown with the specific histochemical staining for mannose because they are covered by Type-1, mannose-sensitive fimbriated bacteria such as *E. coli* or other endogenous ligands. However, these could be displaced by long-term exposure to diets containing mannose-specific lectins such as GNA, thus revealing the presence of mannosyl terminals whose absence in normal rats is therefore only apparent and not real (Figure 1a, b).

FIGURE 1



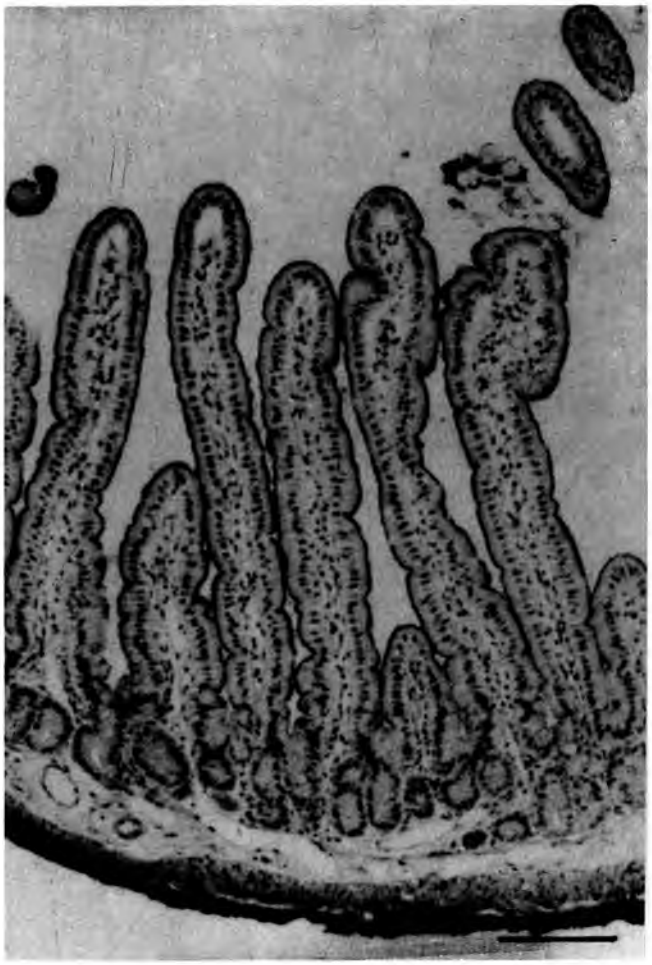


(a) The binding of the *Galanthus nivalis* agglutinin (GNA) is slight on acute exposure but (b) strong binding develops after dietary exposure to this lectin for 10 d indicating the uncovering of mannosyl terminals in epithelial cell membranes. Small intestinal sections were first reacted with monospecific anti-GNA rabbit antibodies, followed by peroxidase-labelled secondary antibodies and development of the colour reaction with 3,3'-diaminobenzidine. Bar = 100 μm.

b. *Changes in glycosylation resulting from increased rate of crypt cell proliferation.*

By speeding up cellular turnover, lectins with powerful growth factor activity can increase the proportion of less differentiated, immature epithelial cells on the villi, with the consequent increase in the concentration of terminally mannosylated cytoplasmic glycoconjugates. Changes in glycosylation can also result from other lectin-stimulated metabolic effects which either increase or decrease the cellular concentration of cytoplasmic and/or membrane glycoconjugates with different terminal glycosyl groups (Figure 2a, b).

FIGURE 2

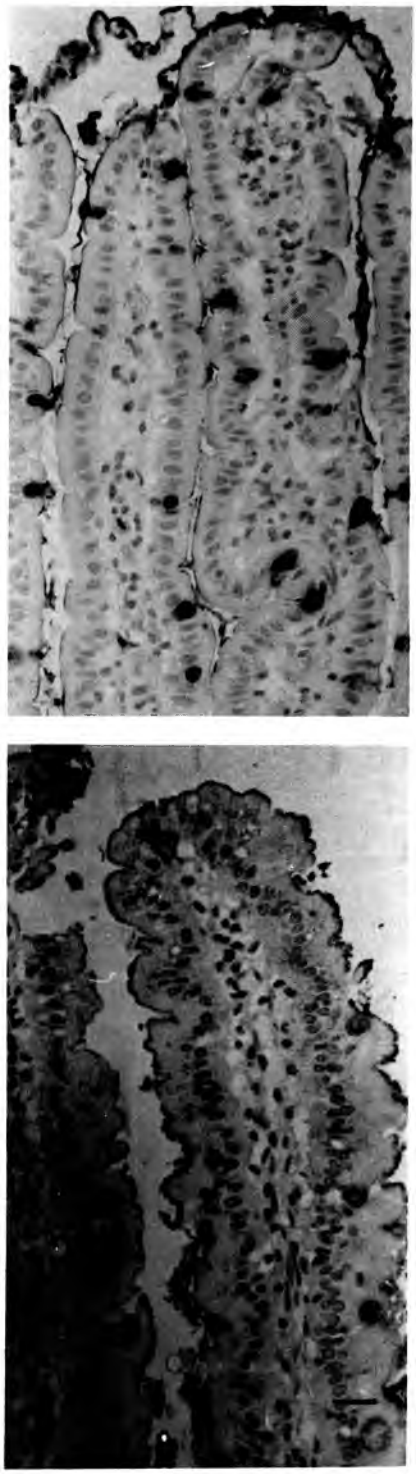


Cartoon of the position of cells on small intestinal villi containing polymannose-type cytoplasmic glycoproteins in rats fed (a) control and (b) PHA-diets. The darker the colour, the more polymannose residues are shown by digoxygenin-labelled GNA.

c. *Changes in glycosylation resulting from the effects of lectins on goblet cells.*

The contents of the goblet cells of both germ-free rats and those with a conventional microflora can nearly be emptied by PHA exposure (Figure 3a, b). In contrast, GNA has the opposite effect. Moreover, exposure to sialic acid-binding lectins can also change the glycosylation of the gut wall by overstimulating and exhausting the mucin synthesizing capacity of goblet cells (2).

FIGURE 3



Staining with digoxigenin-labelled *Aleuria aurentia* agglutinin (specific for α-1,6 fucose) of a section of small intestinal villi of (a) control and (b) PHA-fed rats. There is strong staining of goblet cells in (a) whereas the mucin content of these cells is much depleted in (b). Bar = 25 μm.

Future perspective and practical implications

As dietary lectins induce changes in the glycosylation of brush border membranes or cytoplasmic glycans with the appearance of new or the removal of existing glycosyl structures on the surface of the gut, the binding potential of the small intestinal epithelium for dietary or endogenous factors and bacteria can be radically altered. Since the most critical step in the infection of the gut by bacteria is their attachment to its surface glycosylated receptors through fimbrial- and/or surface adhesins, considerable shifts in the bacterial population of the intestinal tract may be induced by changing the expression of the sugar structures on the luminal surface. Furthermore, as sugar-specificity determines which lectin can bind to the brush border endocrine cells, lectins can also modulate the secretion of gut peptide hormones such as cholecystokinin, gastrin, glucagon and others because, by changing the state of glycosylation of brush border cells, the secretion of these hormones is stimulated or depressed (1,8). By inducing the growth of the gut or modulating the content and/or specific activity of the brush border enzymes in the small intestine or by stimulating the synthesis and secretion of pancreatic enzymes (1,4), lectins can be used to change the physiology and the digestive/absorptive functions of the gut. Some lectins may also be used to stimulate the secretion of mucinous glycoproteins from small intestinal goblet cells. Alternatively, anti-mitotic lectins or incomplete mitogens can be used to slow done or stop unwanted cell proliferation in the gastrointestinal tract. Thus, using dietary lectin(s) it is now possible 'to engineer' the digestive tract for improved performance and bacterial ecology (8). Moreover, as interactions between lectins and gut mucosa are on a solid chemical foundation and predictable, their dietary and medical uses are expected to grow in future.

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Effect of phytohaemagglutinin on intestinal cell proliferation Role of polyamines

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SUMMARY. The polyamines, putrescine, spermidine and spermine, mediate the effects of hormones and growth factors as second messengers. They are necessary for every step of protein, RNA and DNA synthesis and are therefore essential for cell growth and proliferation. As with hormones and peptide growth factors, plant lectins which bind to cell surface receptors of the brush border membrane are powerful extraneous growth factors for the gut and as a result, by interacting with brush border epithelial receptors, induce extensive proliferation and changes in the metabolism of epithelial cells via activation of second messenger pathways. In model experiments with phytohaemagglutinin (PHA, the lectin from the kidney bean, *Phaseolus vulgaris*) it was shown that lectins which bind avidly to the mucosal surface induce dose- and time-dependent and fully reversible hyperplastic and hypertrophic growth of the small bowel. The resulting increase in crypt cell proliferation (CCPR) alters the gene expression in epithelial cells. These metabolic changes require vast amounts of polyamines, mostly spermidine. Thus, one of the first effects of the PHA signal is to stimulate the basolateral polyamine uptake system for the sequestration of polyamines from blood circulation in sufficient amounts to sustain the growth of the tissue. Our data indicate that the main source of polyamines to replenish those taken up by the gut is the diet.

It has been shown repeatedly that, because of intensive cell proliferation, tumour growth requires large amounts of polyamines. PHA or other lectins accelerate normal metabolic reactions in a regulated way while maintaining their full reversibility and without causing irreversible aberrations. The fact that the lectin-induced growth of the gut requires large amounts of polyamines, suggests that lectins are ideal agents to limit the availability of polyamines for unwanted growth such as neoplastic proliferation of tumours. Accordingly, it may be possible to limit the availability of polyamines for tumour growth by inducing a competitive, but fully reversible, controlled growth of the gut tissue. The intensively but reversibly growing gut tissues can slow down tumour growth by sequestering polyamines and nutrients from circulation.

RESUMEN. Efecto de la fitohemaglutinina sobre la proliferación de las células intestinales. Papel de las poliaminas. Las poliaminas, putrescina, espermidina y espermina, actúan como segundos mensajeros mediando los efectos de hormonas y factores de crecimiento. Ellas se requieren en cada paso de la síntesis de DNA, RNA y proteínas y, por lo tanto, son esenciales para el crecimiento y la proliferación celular. A través de su unión a los receptores de la superficie del borde en cepillo, las lectinas de plantas actúan como potentes factores exógenos de crecimiento del intestino y en consecuencia inducen una intensa proliferación celular y cambios en el metabolismo de las células epiteliales por intermedio de la activación de las rutas de los mismos segundos mensajeros. Usando fitohemaglutinina (PHA, la lectina de *Phaseolus vulgaris*), se encontró que aquellas lectinas que se unen ávidamente a la mucosa inducen el desarrollo hipertrófico reversible del intestino delgado, de una manera que es dependiente de la dosis y del tiempo de exposición. El aumento en la proliferación de las células de las criptas (CCPR) altera la expresión de los genes en las células epiteliales, para lo cual se requiere de grandes cantidades de poliaminas, en especial de espermidina. En consecuencia, uno de los primeros efectos de la PHA estriba en incrementar la captación, a través de la membrana basolateral, de poliaminas provenientes de la circulación sistémica, en proporciones suficientes para mantener el crecimiento del tejido. Nuestros resultados indican que la mayor parte de las poliaminas, necesarias para cubrir el déficit producido por el incremento de la demanda intestinal, son de origen dietario.

Se ha demostrado repetidamente que, a causa de su intensa tasa de proliferación celular, los tumores requieren de un suministro incrementado de poliaminas, pudiendo establecerse un proceso de competencia con tejidos normales, que sean estimulados reversiblemente a proliferar, de manera controlada, con PHA u otras lectinas. Por ejemplo, el crecimiento intestinal inducido por lectinas, el cual demanda altas cantidades de poliaminas, sugiere que las lectinas podrían actuar como agentes ideales para limitar la disponibilidad de poliaminas y otros nutrientes de la circulación, disminuyendo, en consecuencia, el crecimiento tumoral.

INTRODUCTION

The epithelium of the small bowel is composed of a monolayer of epithelial enterocytes fulfilling the absorptive and digestive functions of the gut. The epithelium of the intestinal tract has one of the highest cell turnover rates in the body of mammals (1) enabling it to react rapidly to any changes, from dietary influence to bacterial invasion.

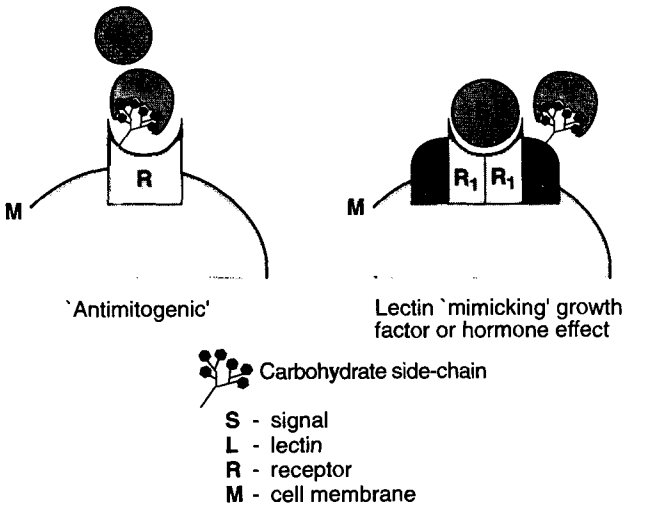
The small intestinal epithelium is organized into two functionally and morphologically distinct compartments: the crypts, where the stem cells proliferate and differentiate, under the influence of growth factors, hormones and cytokines, and the villi, where the differentiated cells mature and migrate toward the tip of the villi for absorption and digestion to occur (1). All cell-surface proteins are glycosylated in order to be transported from the site of synthesis to the plasma membrane (2,3). The pattern of glycosylation varies in different species (4), but within any one species it depends on the stage of differentiation and maturation, on the position of the cells along the crypt-villus axis, on the site of the gastrointestinal tract and also on age and blood group specificity. The great variability in glycosylation may help to explain why lectins differ in their ability to interact with the surface of gut.

Lectins are very potent exogenous growth signals, some can even mimic the action of major metabolic hormones and growth factors. In contrast to dietary proteins which are rapidly degraded during passage through the gut, lectins resist degradation by proteolytic enzymes and also by different bacteria. The almost complete survival of the lectins of *Galanthus nivalis* (GNA), despite the absence of binding to the brush-border in acute exposure, and also of PHA suggests that the molecular structure of some lectins may be intrinsically resistant to proteolytic breakdown (5).

Since most growth factor- and hormone-receptors are glycoproteins or glycoconjugates embedded in the cell surface membrane, interaction between lectins of plant or bacterial origin and the gut depends on specific recognition by the lectin of the membrane glycans projected into the gut lumen. Therefore, the growth factor or hormone-like action of lectins can be explained by their lectin function: through recognition and binding to surface membranes, they send signals and deliver messages into the cells (Figure 1). Receptor proteins are usually composed of more than one subunit, and the subunits exposed on the external side of the membrane are glycosylated.

The process of recognition between lectins and their receptors is instantaneous. The strength of lectin binding is also dependent on the number of unoccupied receptor sites. If there are many carbohydrate side-chains with the 'right' sugar structure the lectin will bind extensively, but if there are just a few binding sites, or the sites are well separated from each other, only weak or no binding occurs. There is evidence that strong binding lectins are readily endocytosed or transcytosed although the signals necessary for these processes are not well understood at the moment.

FIGURE 1

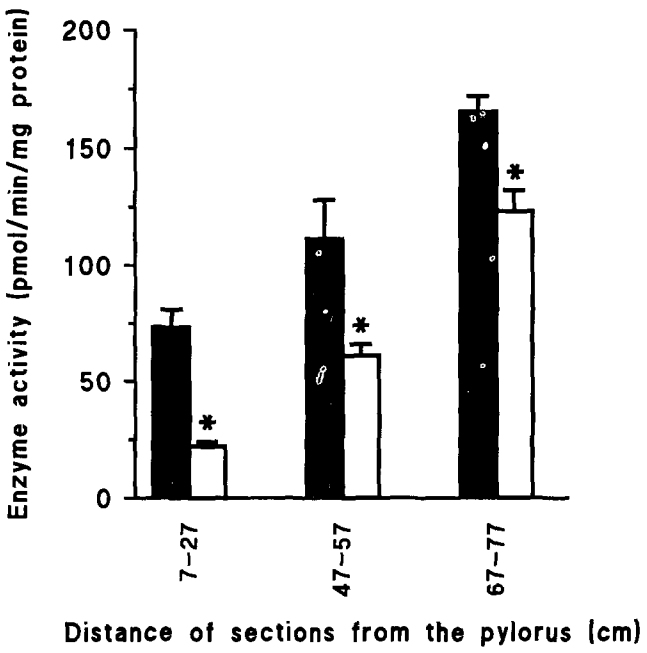


Location of the glycosyl side-chains on the cell-surface proteins in the membrane, and possible interactions of lectins and growth factor- or hormone receptors. Depending on their position, the sugars-structures can be present in or near to the active centre, on the same subunit where the growth factor or hormone-binding site is located or, on another subunit. Accordingly, lectins can bind to the main or other subunit(s) through their glycosyl side-chains and activate them by changing their conformation. These changes can mimic partly or fully the changes induced by the 'proper' biological signal (growth factors, hormones, cytokines, etc...), or, if the carbohydrate side-chain is on another subunit from the signal binding site, they can even attenuate the effects of the signals. In some cases the lectin, by binding to the receptor, can inhibit either the binding of the signal, or prevent some conformational changes in the receptor or in the surrounding membrane domain. These lectins are called 'anti-mitogenic.'

Some carbohydrates are scanty in the small bowel, but present in the large intestine so that lectins which are specific to those receptors will pass through the small bowel and bind to the surface of the large intestine. As a result, most lectins can also affect the metabolism of the large intestine. The growth factor activity of gut lectins is determined mainly by the strength and intensity of their binding (5). Even when this is relatively weak, by cross-linking cell surface receptors it still disturbs the organization of the epithelial membrane (5,6) and induces slight growth of the gut. PHA is a potent growth factor for the mammalian gut and is the best studied model. On lectin-induced small intestinal growth, the length of the villus is rarely significantly affected (5). In contrast, the crypt size, the number of cells they contain and the CCPR are substantially increased (7). These changes correlate well with the effectiveness of the lectins as growth factors. However, the newly produced cells need time to differentiate, and since the migration speed of cells on the villus is also faster, the proportion of immature cells on the villi rises. With continuous

exposure to lectins such as PHA, which binds extensively, the cell turnover time can decrease from 72 to 12 hours. As protein and enzyme patterns of the newly formed cells are typical of the immature cell type, there are significant differences in the capacity of those cells to absorb and digest food. On exposure to PHA, there is a significant increase in the activity of maturation marker enzymes such as diamine oxidase in the gut tissue (Figure 2) and the specific activity of sucrase-isomaltase and alkaline phosphatase (8).

FIGURE 2



Diamine oxidase activity in the small intestine of rats fed either lactalbumin (■) or 42 mg PHA/rat/day (□) diets for 10 days. Values are means ± SE of 9 rats. $P<0.01$ (*) when compared to control (lactalbumin) values.
(These data were produced in collaboration with Drs Perin and Sessa, Universita Delgi Studi di Milano, Italy).

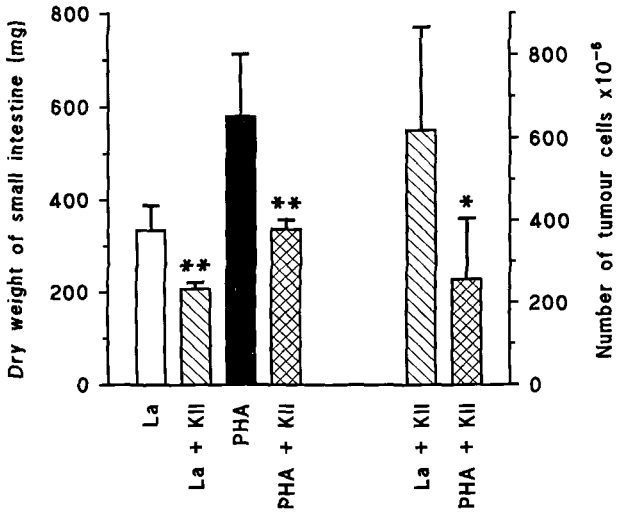
Even a small increase in the size of the gut has a slight nutritional penalty for the animal, in that more of the dietary protein and energy are consumed in the need to renew the gut surface more quickly than under normal conditions. With PHA and the lectins from soya bean (*Glycine max*, SBA) or wheat germ (*Triticum vulgaris*, WGA) which bind avidly to epithelial cells and are more powerful growth factors for the small bowel, the cost in nutritional terms is even more expensive. Indeed, at high dietary intakes of these lectins, most or all of the diet is used by the gut alone leaving other organs starved of nutrients (9,10). Diets containing PHA or raw kidney beans can double the weight of the small intestine within 7 days (11-14). However, the contribution of the growth stimulating activity of the lectins to their nutritional toxicity is relatively slight under most practical conditions.

The most studied example is PHA which recognises and binds to the complex carbohydrate structures present on the intestinal surface of mammals. One of the first changes induced in the epithelium of the small intestine of the rat is the instant stimulation of protein synthesis (15,16). The second and long-lasting increase in protein synthesis is evident after a few hours and results in the synthesis of new RNA (17). These processes require the polyamines putrescine, spermidine and spermine (18,19) which are essential for the adaptational growth of the gut (20,21). The growth of the gut induced in conventional rats by PHA requires the accumulation of large amounts of polyamines, mostly spermidine, in the tissue (17). However, this accumulation occurs without a major increase in the activity in the small intestine of ornithine decarboxylase (ODC), the rate-determining enzyme of polyamine synthesis (11,17) indicating that, in this instance, ODC has little to do with the increase in polyamine concentration. In the growing intestine, part of the polyamine pool originates from the systemic circulation through the basolateral membrane and one of the first steps of this process is the stimulation of the basolateral uptake of polyamines (11,22). Therefore, measurement of the tissue polyamine content and the rate of basolateral uptake of polyamines, mostly spermidine, can be used as markers of the metabolic activity of the intestine and to follow the effects of different antinutritional factors, including the growth factor-like effects of the lectins on gut metabolism.

The recognition that the lectin-induced changes in cellular metabolism are fully reversible has allowed us to use the PHA-induced rat small intestinal growth model as a convenient tool for magnifying and studying the fundamental metabolic reactions of epithelial cell proliferation, differentiation and maturation and ensuing changes in the cellular metabolism of the gut. In recent developments, PHA was used to manipulate body metabolism of tumour-bearing mice with the aim of redirecting nutrients and polyamines away from the tumour by a competing growth signal. PHA is ideal for such an experiment since it induces reversible, polyamine-dependent, hyperplastic growth of the small bowel (11) and has been shown to compete successfully with hypertrophic growth of the skeletal muscle induced by clenbuterol, a ß-adrenoreceptor agonist (16). It was thought, therefore, that PHA might also compete with the putative tumour growth signal(s) and stop, or at least slow down neoplastic proliferation. We have shown that NMR mice fed PHA-containing diets do indeed have fewer tumour cells than the lactalbumin fed control animals, when injected intraperitoneally with Krebs-II ascites cells. As expected, in the PHA-fed animals the weight of the gastrointestinal tract increased while the number of tumour cells in the peritoneum decreased (Figure 3). Earlier observations (23,24) and also evidence presented in Figure 4 show that our approach based

on interorgan competition is valid as PHA was able to slow down the proliferation of Krebs-II ascites cells. Biochemical analysis of the tissues (protein, RNA, DNA and polyamine contents) indicated that interorgan competition between the tumour and vital organs can be used to manipulate the metabolism of tumour-bearing mice, or compete with any unwanted growth.

FIGURE 3



Dry weights of the small intestine and number of tumour cells of tumour-bearing mice fed either on kidney bean (PHA) or control lactalbumin (La) diets. The values and mean ± SD for 5 animals per treatment. ** Significant difference between those mice injected with Krebs-II ascites cells (KII) are their respective controls ($P<0.001$). * Significant difference in number of tumour cells between those mice fed PHA diets and the La controls ($P<0.05$).

ACKNOWLEDGEMENTS

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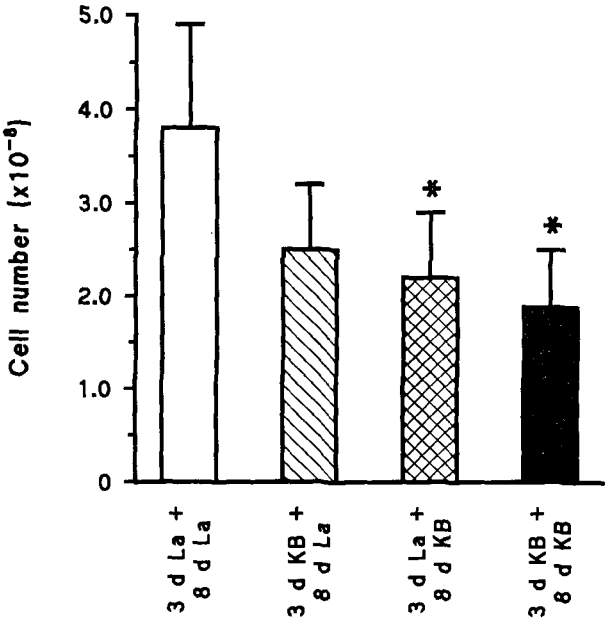
FIGURE 4



Four different groups of NMR mice were injected intraperitoneally with Krebs-II ascites cells. For 11 days each group followed a different combination of the lactalbumin (control) and/or PHA (42 mg/mouse/day) diets. Values are means ± SD of 5 mice. $P<0.05$ (*) when compared to control values.

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Interaction of proteases with legume seed inhibitors. Molecular features [1]

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SUMMARY. After having found that raw black beans (*Phaseolus vulgaris*) were toxic, while the cooked ones constitute the basic diet of the underdeveloped peoples of the world, in the sixties, our research directed by Dr. Jaffé, concentrated mainly around the detection and identification of the heat labile toxic factors in legume seeds. A micromethod for the detection of protease inhibitors (PI) in individual seeds was developed, for the purpose of establishing that the multiple trypsin inhibitors (TI) found in the Cubagua variety were expressions of single seeds and not a mixture of a non homogenous bean lot. Six isoinhibitors were isolated and purified, all of which were «double-headed» and interacted with trypsin (T) and chymotrypsin (CHT) independently and simultaneously, as shown by electrophoresis of their binary and ternary complexes with each and both enzymes. However, their affinity for the enzymes, including elastases, was rather variable, as well as their amino acid composition which consisted of 51 units for inhibitor V, the smallest, and 83 amino acids for inhibitor I, the largest.

A low molecular weight protein fraction that inhibited subtilisin (S), but recognized neither T, CHT nor pancreatic elastase was detected in 63 varieties of *Phaseolus vulgaris* as well as in broad beans (*Vicia faba*), chick peas (*Cicer arietinum*), jack beans (*Canavalia ensiformis*), kidney beans (*Vigna aureus*),etc., It was absent though, in soybeans (*Glycine max*), lentils (*Lens culinaris*), green peas (*Pisum sativum*), cowpea (*Vigna sinensis*) and lupine seeds (*Lupinus sp*).

Subtilisin inhibitors (SI) were isolated from black beans, broad beans, chick peas and jack beans. Their Mr is between 8-9KD and they show a rather high stability in the presence of denaturing agents. They are specific toward microbial proteases, in addition to subtilisins, Carlsberg and BPN', they inhibit the alkaline protease from *Tritirachium album* (Protease K), from *Aspergillus oryzae* and one isolated from *Streptomyces griseus*, but do not interact with either animal digestive or plant thiol enzymes.

RESUMEN. Interacción de proteasas con inhibidores de semillas de leguminosas. Caracterización molecular del proceso. Luego de encontrar que las semillas crudas de *Phaseolus vulgaris* eran tóxicas, mientras que las cocidas eran la base de la dieta en muchos países en vías de desarrollo, en los años sesenta nuestras actividades de investigación bajo la supervisión del Dr. Jaffé se centraron en la detección e identificación de factores de naturaleza termolábil en las semillas de leguminosas. Se desarrolló un micrométodo para la detección de inhibidores de proteasas en semillas individuales. Se encontró que las múltiples formas de los inhibidores de tripsina (TI)encontradas en la variedad Cubagua constituían en realidad isoformas y no eran el producto de un lote de semillas no homogéneo. Se aislaron y purificaron seis isoinhibidores de «doble cabeza» capaces de interactuar con la tripsina (T) y la quimotripsina (CHT)de manera simultánea e independiente, lo cual se evidenció aislando los complejos binarios con cada enzima y los ternarios con ambas proteasas. Sin embargo, su afinidad por las enzimas, incluyendo las elastasas fue muy variable, así como también su composición de aminoácidos, desde 51 en el isoinhibidor más pequeño (V) hasta 83 en el más grande (I).

Una fracción proteica de bajo peso molecular, capaz de inhibir a la subtilisina pero que no reconocía a T ni a CHT ni a la elastasa pancreática, fue detectada en 63 variedades de *P. vulgaris*, así como en habas (*Vicia faba*), garbanzos (*Cicer arietinum*), haba de burro (*Canavalia ensiformis*), frijoles (*Vigna aureus*), etc. No se encontró el inhibidor en otras especies como la soya (*Glicine max*), lentejas (*Lens culinaris*), frijoles (*Vigna sinensis*) y lupino (*Lupinus sp*).

El inhibidor de subtilisina (SI) fue aislado de caraotas, habas, garbanzos y habas de burro. Sus Mr se encuentran entre 8-9 KD y tienen una gran estabilidad frente a agentes denaturalizantes. Los SI mostraron afinidad por proteasas microbianas, además de las subtilisinas Carlsberg y BPN, ellos inhibieron a la proteasa alcalina de *Tritiraquium album* (Proteasa K), de *Aspergillus oryzae* y una aislada de *Streptomyces griseus*, pero no interactuaron ni con las enzimas digestivas de animales ni con las tiólicas de plantas. Los SI reaccionaron con S a través del «mecanismo convencional» de interacción de T con TI. Este enfoque fue usado para identificar el sitio reactivo del SI de caraotas negras como un enlace ALA-LEU(ILE); se encontró,

[1] This paper is dedicated to Dr. Werner G. Jaffé and was presented at a symposium organized to celebrate his 80th birthday. It covers part of the work I had the privilege and the pleasure to share with the Maestro in the last 30 years.

SI react with S by the «standard mechanism» proposed for the interaction of T with TI, and their reactive sites are split and resynthesized as in TI. This latter method was used to identify the reactive site of black bean SI against S as an ALA-LEU(ILE) bond and a LYS-VAL bond split by TPCK-trypsin, the target enzyme of which is still unknown. Structural differences among the SI isolated from different legume species were suggested by variations in specific activities, in stability under denaturing conditions and mainly in immuno-chemical assays.
The nutritional significance of bean TI was focused on by developing alternative methods to detect their toxicity.

además, un enlace LYS-VAL roto por la TPCK-tripsina, cuya enzima blanco se desconoce. La variación en la actividad específica, su estabilidad frente a agentes desnaturalizantes y los resultados de pruebas inmunoquímicas sugieren diferencias estructurales entre los SI aislados de varias leguminosas.
La significación nutricional de los TI de caraotas fue ensayada usando métodos alternativos a fin de determinar su toxicidad.

For enzymes to interact with substrates structural compatibility is a necessary requirement. Proteases degrade proteins into peptides according to the specificity of the enzyme. Natural protease inhibitors are proteins whose structure is recognized but not degraded by the enzyme. They form complexes with the latter, blocking it from interacting with substrate molecules.

This paper is limited to the discussion of the relations of two animal proteolytic enzymes: trypsin (T) and chymotrypsin (CHT) and a bacterial one: subtilisin (S), with their natural inhibitors isolated in our laboratory from legume seeds.

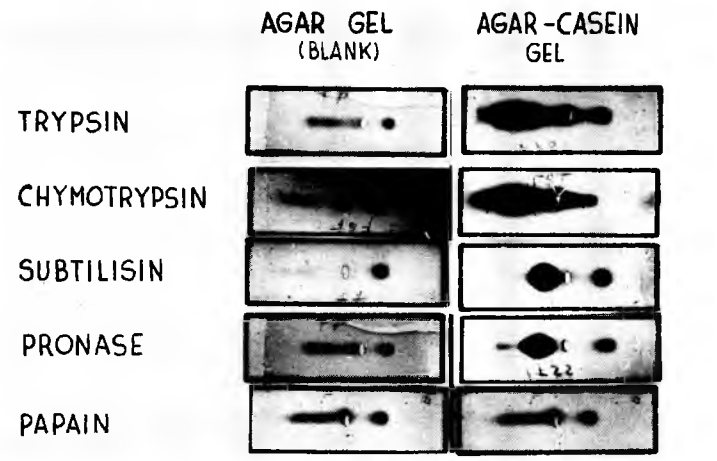
In the sixties, Osawa and Laskowski demonstrated that the interaction of T with the Kunitz soybean inhibitor and with chicken ovomucoide involves the cleavage of a single peptide bond of the arg-x or lys-x type, which affects their activity. Based on these initial observations the authors proposed that one of these bonds on the molecular surface of the natural inhibitors constitutes the site of interaction with the enzyme [1].

Both method and the «reaction mechanism» hypothesis started many of the researchers assisting at this meeting, to look into the molecular interaction of proteases with their inhibitors, and into the study of the reactive sites of those isolated from soybeans (*Glycine max*), black and garden beans (*Phaseolus vulgaris*), broad beans (*Vicia faba*), jack beans (*Canavalia ensiformis*), etc.

In our laboratory, at the Universidad Central de Venezuela, the research efforts concentrated around the study of those heat-labile toxic factors from black beans (*Phaseolus vulgaris*), that inhibit proteolytic enzymes. Seven T inhibitory fractions were detected in aqueous extracts of the cv Cubagua on disc gel electrophoresis. It was necessary to elucidate whether these activities were due to a mixture of inhibitors originating from different seeds or to isoinhibitors, the genetic expression of each single seed. In order, to find the correct answer the development of a micro-method capable of detecting inhibitors in single seeds was required.

At that time, Eglis González, Dr. Jaffé's student, and Antonio Callejas, the best technician we ever had, started to screen 12 legume species for proteolytic enzyme inhibitors [2]. The method used consisted of electrophoretic separation of seed protein extracts on agar-casein gel plates. The gels were then covered with filter paper slips soaked in a protease solution, which during incubation digested casein, except in the areas where inhibitor(s) blocked degradation. These spots became visible after coloring with amido black (figure 1).

FIGURE 1
Electrophoretic and inhibition patterns of protease inhibitors on agar gel plates



The method was then adjusted to the microgram scale and the bean lots were analyzed seed by seed. The results inferred that the lots were homogeneous and the T inhibitors (TI) in multiple forms were present in each and every seed [3].

The screening of 63 varieties of Phaseolus vulgaris showed the omnipresence of TI. However, their migration and inhibition patterns varied greatly within the species (figure 2), (2).

Six isoinhibitors were purified to electrophoretic homogeneity from cv cubagua their specificity spectra (table I), amino acid composition (table II) and active sites against T and CHT were determined. All inhibitors showed to be double headed, having independent reactive sites. Their binary complexes with either T or CHT and a ternary complex with both enzymes were identified on acetate cellulose membrane after electrophoresis (figure 3).

FIGURE 2
Variability of protease inhibitors in single *Phaseolus vulgaris* seeds

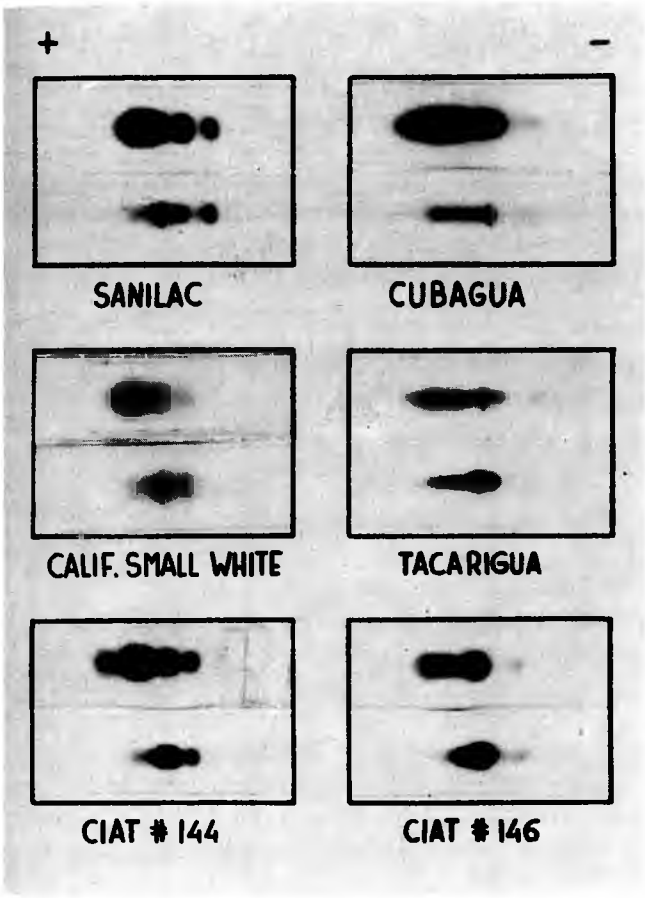


FIGURE 3
Acetate cellulose electrophoresis of black bean trypsin inhibitor (ITC_1) complexes with trypsin (lanes 3 and 4), chymotrypsin (lane 7) and both enzymes (lanes 5 and 6)

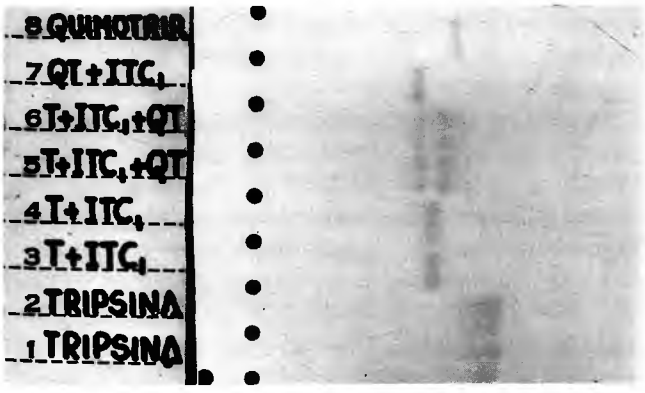


TABLE I
SPECIFICITY SPECTRUM OF BLACK BEAN ISOINHIBITORS

INHIBITOR #	ug I*/nmol T	ug I*/nmol CHT	ug I*/nmol HLE	ug I*/nmol BPE
I	14.5	64.0	14.1	16.8
II	12.0	11.0	23.3	85.0
III	7.1	7.8	12.5	47.5
IV	3.0	9.2	9.2	11.0
V	4.5	8.3	14.6	26.0
VI	4.2	8.9	14.6	26.0

* Micrograms of inhibitor required to inhibit 1 ug of the indicated enzyme. BAPNA was used as substrate for trypsin (T), GPNA for chymotrypsin (CHT) and Succ-(ala)3-p-NA for human leucocyte elastase (HLE) and bovine pancreatic elastase (BPE).

TABLE II
AMINO ACID COMPOSITION OF BLACK BEAN ISOINHIBITORS

AMINO ACID	INHIBITOR					
	I	II	III	IV	V	VI
LYS	5	5	3	3	3	3
HIS	2	3	2	2	2	2
ARG	3	2	2	2	2	2
ASP	7	8	6	7	7	7
THR	7	5	3	4	3	4
SER	13	12	8	8	7	8
GLU	8	8	5	5	5	5
PRO	6	6	5	4	4	4
GLY	10	5	1	3	1	1
ALA	5	5	3	3	3	2
CYS	4	8	8	8	8	10
VAL	2	1	-	1	-	-
MET	1	-	-	-	-	-
ILE	3	3	3	2	2	3
LEU	4	3	2	2	2	2
TYR	2	2	1	2	1	2
PHE						
TOTAL	83	78	54	57	51	55

During the isolation of TI from black beans, Selma Olivares and later Fanny Locker, two of my thesis students, found in the eluate of the bean extract, on an ion exchange column a low

molecular weight protein fraction that inhibited subtilisin, but recognized neither T, CHT nor pancreatic elastase [4]. The subtilisin inhibitor (SI) activity was also detected on agar-casein gel electrophoresis in the 63 varieties of phaseolus vulgaris mentioned earlier, as well as in broad beans (*Vicia faba*), chick peas (*Cicer arietinum*), jack beans (*Canavalia ensiformis*), kidney beans (*Vigna aureus*),etc., It was absent though, in soybeans (*Glycine max*), lentils (*Lens culinaris*), green peas (*Pisum sativum*), cowpea (*Vigna sinensis*) and lupine seeds (*Lupinus sp*)[2].

The first SI was isolated from black beans (cv. Cubagua) in collaboration with Hugo Abreu. It contains 82 amino acids, has two disulfide bridges and a Mr of 9KD. Its stability in the presence of denaturing agents is similar to that of TI from legumes [4].

The inhibitors purified later on, with Juscelino Tovar, Pilar Lorenzo and Elena Pinelli, from chick peas, broad beans and jack beans have similar molecular weights and show the same characteristic stability with only slight variations. For example, the chick pea inhibitor is the only one denatured by 5% TCA at room temperature, while all others are resistant [5,6].

We named the above inhibitors SI because commercially available subtilisin Carlsberg was used to detect and estimate them, just as bovine T is used in the TI assays. It is clear though, that in neither case the enzyme is necessarily the principal target of the inhibitor.

SI studied in our laboratory are rather specific toward bacterial serine proteases. In addition to subtilisins, Carlsberg and BPN', they inhibit the alkaline protease from *Tritirachium album* (Protease K), that of *Aspergillus oryzae* and one isolated from *Streptomyces griseus*. Inhibition level varies, however, from one seed species to the other indicating structural differences between the inhibitors. These apparently do not interfere with enzyme recognition, but can affect negatively the stability of the enzyme-inhibitor complex. It could cause an increase in the dissociation constant of the complex and in the amount of inhibitor required for complete inhibition [7].

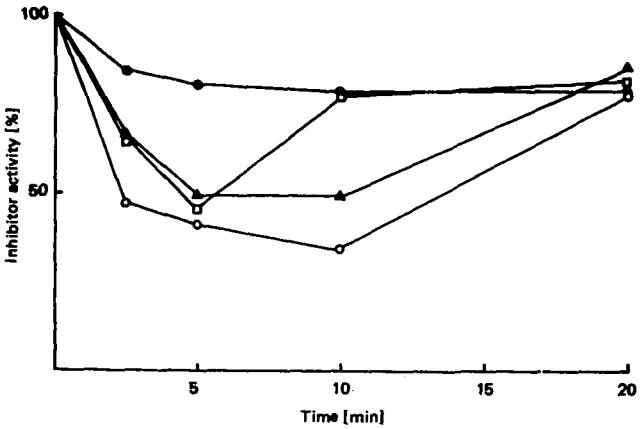
An unusual feature was detected in the chick pea SI, namely that it recognizes the structural differences existing between subtilisin Carlsberg and BPN'. It inhibits the former six times stronger, probably due to the higher dissociation constant of the BPN'-SI complex [5].

Specificity studies of the inhibitors indicate that no interactions occur with either CHT, or plant thiol enzymes, or microbial acid proteases. Their capacity to inhibit T was slight or not detectable [6]. However, a «silent» reactive site against T was found to be present in the black bean SI [8].

Using the Laskowski method for the identification of active sites on inhibitor surfaces, it was shown that the «anti-subtilisin» site of the black bean inhibitor is ala-leu(ile) bond. When SI is modified (SIs) by selective hydrolysis of this bond it loses activity, which is restored upon resynthesis [8]. Figure 4 depicts the modification of four inhibitors by S as a function of incubation time. Activity decreases at the begining of the period and tends to increase, reaching an equilibrium at about 80% of the original value after 20 min. This pattern suggests that SI react with S according to the «standard reaction mechanism» proposed by Osawa and Laskowski [1] for the interaction of T with its natural inhibitors.

FIGURE 4

Reactive site modification of subtilisin inhibitors. *Phaseolus vulgaris* (●), *Cicer arietinum* (Δ), *Canavalia ensiformis* (○) and *Vicia faba* (□). Cleavage of the active site was performed under the following conditions: Subtilisin: inhibitor molar ratio 1:1, pH 8 and dissociation of the complex in 15% acetic acid.



In order to be able to compare the splitting of the active site bond the original method had to be modified. Instead of catalytic amounts of S, equimolar enzyme:inhibitor ratio was applied, and the acid incubation medium was changed to pH 8. Under these conditions the bond on all four inhibitors was split and resynthesized. Due probably to structural differences, modification with S of the chick pea SI occurs only at neutral or alkaline pH, while that of black bean preferably in acid solution; those from broad beans and jack beans react at both pH values.

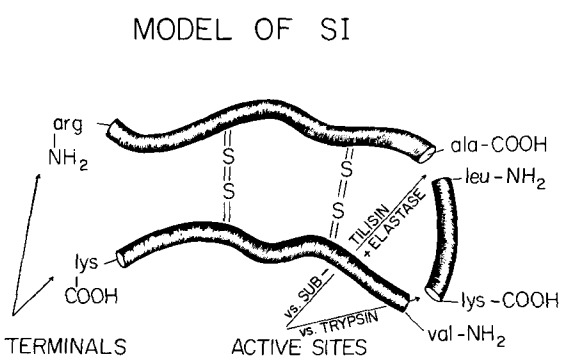
Two interesting molecular features of the black bean SI should be recalled. Although it does not inhibit pancreatic elastase, it interacts with human leucocyte elastase (HLE) in a 1:1 molar ratio. This inhibition is reverted by adding increasing amounts of S to the incubation mixture, suggesting that both enzymes compete for the same reactive site of SI [8].

The second unusual property was found when the black bean SI was incubated with catalytic amounts of TPCK-trypsin and a lys-val bond was split. The T modified SI (SIt) loses some S inhibitory activity. However, when the new lys terminal is cleaved by carboxypeptidase B, activity is abolished. Considering the fact that black bean SI shows only a very slight activity against T, it was concluded that the molecule possesses a «silent» reactive site recognized by T [8].

It is impossible to conclude whether the two reactive sites of the black bean SI overlap or are independent, for the

«target» enzyme for the «trypsin-site» has not yet been found. They might be close to one another, because cleavage of lys from the «trypsin-site» inactivates interaction with S. Alternately, lys may be important for the formation of the S-SI complex, or else due to the presence of only 2 disulfide bonds per molecule, as compared to 7 in the Bowman-Birk inhibitor, conformational changes occur upon lys cleavage causing the observed inactivation. Based on these data a structural model for the black bean SI has been proposed (figure 5).

FIGURE 5
Hypothetical model of black bean subtilisin inhibitor, indicating active sites, terminals and disulfide bonds



When the four SI inhibitors isolated from different legume species were further compared, immunochemical differences were detected. Antibodies raised against jack bean SI, which recognized SI from other species and varieties of the Canavalia genus, did not interact with the inhibitors purified from either black beans, broad beans or chick peas.

In spite of their structural differences and based on similarities in specificity, «reaction mechanism», heat stability and molecular weight, we suggested the possibility of classifying the SI from legume seeds into a separate «family» of protease inhibitors [6].

In order to determine the effect of an antinutritional factor in a certain animal, quantities in the order of grams are required. As the amounts of inhibitor present per 100 g seed are on the mg scale, we looked at alternative methods able to predict possible toxic effects on an organism. Based on the hypothesis that the relative sensitivity of a pancreatic protease of an animal to an inhibitor can affect the entire digestive process, we compared the activity of 7 inhibitor preparations on T and CHT from 12 animal species, including bovine and human [9].

Interactions varied widely from one species of animal to another, as well as from one seed species to another. From our in vitro results we could not predict the in vivo nutritional effect of the TI tested. However, it was clearly shown that assessing TI content of a foodstuff with bovine T is an unwise practice, for what can look like low activity with this enzyme may affect seriously the digestive process of an animal from another species fed the TI containing diet [9].

The other alternative for the study of antinutritional effects is the use of small model organisms requiring minute amounts of a purified inhibitor. Several years ago the thesis of J. Szwarcbort, established a correlation between the toxicity of black bean proteins in mice and in rice weevils (*Sitophylus oryzae*). Recently we used this system to determine the toxicity of different antinutritional factors in jack bean flour [10].

The half-life of young adult weevils, on artificial seeds made from peeled dry pea flour, is 183 days, as compared to 5.3 days on jack bean flour seeds and about 10 days without food. Surprisingly, the cooking of the jack bean did not improve survival, suggesting that a synergistic effect of the different toxic factors causes the premature death of the insects.

The addition of TI to the pea meal, in a concentration equivalent to the one in the jack bean seed, caused only a very slight decrease in survival, probably due to an excess of nutrients in the basic pea diet [10].

This hypothesis is supported by the fact that adults have a low amino acid requirement, allowing survival in defatted, deproteinated corn starch. Addition of casein to this basal diet, however, produces an increase in the insect mean body weight. This might be hindered by TI. At the present, the characteristics of the minimal basic diet are being determined, in order to start a systematic study of the effect of purified antinutritional factors even when available only in small amounts.

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Protein proteinase inhibitors in legume seeds -overview

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SUMMARY. Protein proteinase inhibitors are widely distributed in plant seeds, particularly in legumes. The specificity and potency of inhibition depend on defined inhibitory sites and on the animal species of the target proteinase. Feeding experiments on diets containing isolated soybean trypsin inhibitors (the Kunitz soybean trypsin inhibitor STI and the Bowman-Birk trypsinchymotrypsin inhibitor BBI) caused insignificant growth depression in ratas and chicks, but induced enlargement of the pancreas in ratas, chicks and mice but not in pigs, dogs, calves, monkeys and presumably humans. The trypsin-inhibitory site has been responsible for induction of the pancratic enlargement. The trypsin-chymotrypsin inhibitors from soybeans and from chickpeas inhibit insect midgut proteinases, supporting the hypothesis that proteinase inhibitors comprise a built-in defense mechanism of the seed against insects. Findings on the involvement of proteinase inhibitors, such as BBI, in prevention of tumorigenesis suggest a possible positive contribution of the inhibitors to the nutritional value of legume seeds. BBI is also an effective inhibitor of nephrotoxicity induced by the antibiotic gentamicin. BBI does not cause side effects and does affect the antimicrobial activity. The *in vitro* effects of proteinase inhibitors on animals should be interpreted with caution when related to humans.

RESUMEN. Avances en la investigación sobre los inhibidores proteicos de proteasas en semillas de leguminosas. Los inhibidores proteicos de proteasas se encuentran ampliamente distribuidos en las semillas de plantas, particularmente en las leguminosas. La especificidad y potencia de la inhibición depende de sitios definidos en el inhibidor y de la especie animal de donde se obtengan las proteinasas utilizadas. Experimentos nutricionales con dietas que contenían inhibidores purificados de soya (el inhibidor de tripsina de Kunitz, STI, y el inhibidor de tripsina y quimotripsina de Bowman-Birk, BBI) evidenciaron una insignificante reducción del crecimiento en ratas y pollos, pero indujeron hiperptrofia del páncreas en ratas, pollos y ratones, pero no en cerdos, perros, terneras, monos y, presumiblemente, tampoco en humanos. Se ha atribuido al sitio de inhibición para la tripsina la responsabilidad de promover el crecimento del páncreas. Los inhibidores de tripsina-quimotripsina de soya y de garbanzo inhiben las proteinasas del tracto intestinal medio de insectos apoyando la hipótesis de que los inhibidores representan un mecanismo de defensa de las semillas en contra de los insectos. Los hallazgos sobre la participación de los inhibidores de proteasas, tales como el BBI, en la prevención de la tumorogénesis, sugieren una posible contribución positiva de los inhibidores al valor nutricional de las semillas de leguminosas. BBI también es un potente inhibidor de la nefrotoxicidad inducida por el antibiótico gentamicina y, aunque no causa efectos secundarios, si afecta la actividad antimicrobiana del antibiótico. Los efectos *in vitro* e *in vivo* de los inhibidores de proteasas sobre los animales deben ser interpretados con cautela cuando se refieren a los seres humanos.

INTRODUCTION

The increasing interest in protein-rich legume seeds for use in human and animal nutrition is bringing also into perspective the possible, long -and short-term nutritional effects of the endogenous proteinase inhibitors. The inhibitors are proteins with molecular weights in the range of 7 to 25 kd and they amount to 0.1% of the seed protien content. Their physiological role in the plant is still being questioned. The fact that legume seeds contain inhibitors of growth and of gut proteinases of several stored-product insects suggested the hypothesis that the inhibitors have evolved as a defense mechanism against predatory insects. The first trypsin inhibitor from legume seeds was isolated and characterized by M. Kunitz in 1947 (1). By now the presence of proteinase inhibitors in all legume seeds is an established fact. The inhibitors differ in specificity and in capacity to inhibit one or two proteinases at the same time. Several kinds of inhibitors can be present in a single tissue, as in soybeans. The possible antinutritional effects of legume seed proteinase inhibitors have been studied

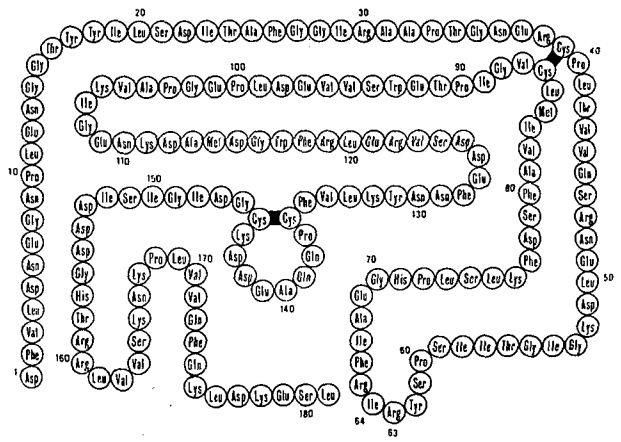
extensively (reviewed, 2). However, the quantification of the effects of proteinase inhibitors *per se* is hampered by several factors such as the form in which the inhibitors are present - raw seeds or purified inhibitors- the feeding strategy and the protein status of the animal (3). The evidence that proteinase inhibitors constitute a hazard to health is often only presumptive since the research relating to its harmfulness has been done with experimental animals consuming large quantities of a particular constituent over a lengthy period of time - conditions quite different from the level of the inhibitor in a normal varied diet.

The epidemiologic evidence for a cancer protective role of proteinase inhibitors in populations consuming legume seeds rich in inhibitors comes primarily from correlation studies (4). The epidemiologic observations led to extensive investigations of proteinase inhibitors as cancer chemopreventive agents of major human cancers (reviewed and summarized, 5). The possible therapeutic contribution of dietary proteinase inhibitors to the prevention of certain types of cancer open a new era in the research of legume seed proteinase inhibitors possible therapeutic contribution of dietary proteinase inhibitors.

EXPERIMENTAL RESULTS

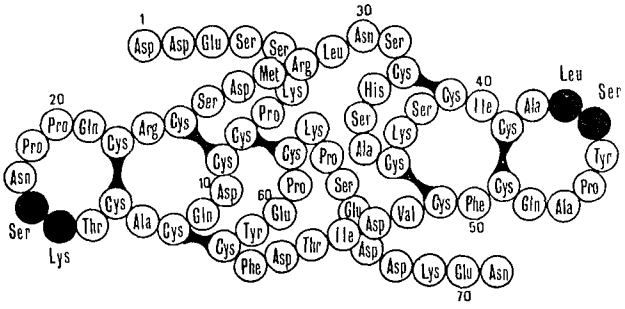
A summary representing the current knowledge on the distribution of legume seed proteinase inhibitors and their specificity of inhibition is given in Table 1 (6,7). The Kunitz soybean trypsin inhibitor (STI), molecular weight~22 kd (1,8), inhibits trypsin *via* the inhibitory site at Arg63- Ile64 (Fig. 1).

FIGURE 1
Amino acid sequence of the Kunitz soybean trypsin inhibitor (8)



It has been also studied thoroughly in numerous laboratories and served also for the establishment of the Standard Mechanism of Inhibition (9). STI is inactivated by heat and by gastric juice (10). However, only a few inhibitors homologous to STI have been found in common legume seeds. The predominant type of inhibitor in legume is the Browman-Birk trypsin-chymotrypsin inhibitor (BBI) from soybeans (Fig. 2) (11,12).

FIGURE 2
Amino acids equence of the Bowman-Birk soybean trypsin and chymotrypsin inhibitor (12)



It has a molecular weight of~8 kd and it forms a 1:1 complex with either trypsin or chymotrypsin and a ternary complex with both enzymes *via* the inhibitory sites at Lys16-Ser17 and Leu43-Ser44 against trypsin and chymotrypsin, respectively. BBI inhibits bovine, porcine, avian, fish and human trypsins and chymotrypsins and is also a potent inhibitor of trypsin - and chymotrypsin-like enzymes from the digestive tracts of insects. The inhibitor is relatively stable to heat and to gastric juice and has unusual resistance to proteases, such as pepsin and pronase. Inhibitors homologous to BBI have been found in lima beans, garden beans, adzuki beans, mung beans, ground nuts and chickpeas (summarized 2, 13, 14). Six proteinase inhibitors have been recently isolated from winter pea seeds. Their partial sequence suggests that they belong to the Bowman-Birk family of trypsin inhibitors (15). Extensive studies have been carried out on lentil seeds, from which twenty-three proteinase inhibitors, belonging to the Bowman-Birk inhibitor family, have been isolated and partly characterized (16). Recently, the expression of BBI and of several active mutants has been achived in *E. coli* (17).

The discovery of thermo-labile trypsin inhibitors in soybeans led to the assumption that the beneficial effect achieved by heat processing of raw soybean meal is due to the destruction of the inhibitors. Feeding experiments of rats and chicks carried out on properly heated soybean meal diets supplemented with STI, BBI, or both, resulted in an insignificant depression of animal growth rate, but the inhibitors were responsible for pancratic enlargement (18). In addition, feeding of rats with raw soybean protein isolates that had a very low trypsin inhibitor content resulted in remarkable growth depression (19). The fachure of soybean trypsin inhibitors to cause growth depression was also demostrated *in calves (20).*

TABLE 1
DISTRIBUTION OF PROTEINASE INHIBITORS PRESENT IN LEGUMES

Botamical name	Common name	Proteinases inhibited[a]
Arachis hypogaea	Peanut, groundnut	T,C,Pl, K
Cajanus cajan	Pigeon pea, red gram	T
Canavalia ensiformis	Jack bean, sword bean	T,C,S
Chamacrista fasiculata	Partridge pea	T
Cicer arietinum	Chick pea, Bengal gram, Garbanzo	T,C
Clittoria ternatea	Butterfly pea	T,C,S
Cyamopsis tetragonoloba	Cluster bean	T,C,S
Dolichos biflorus	Horse gram	T
Dolichos lablab	Hyacinth bean, Hakubenzu bean	T,C, Th
Faba vulgaris	Double bean	T
Glycine max	Soybean	T, C
Lathyrus odoratus	Sweet pea	T
Lathyrus sativus	Chickling vetch	T, C
Lens esculenta (culinaris)	Lentil	T, C
Lupinus albus	Lupine	T
Mucana deeringianum	Florida velvet bean	T
Plaseolus aconitifolius	Moth bean	T
Phaseolus angularis	Adzuki bean	T, C
Phaseolus aureus	Mung bean, green gram	T, endopeptidase
Phaseolus coccineus	Scarlet renner bean	T, C
Phaseolus lunatus	Lima bean, butter bean	T, C
Phaseolus mungo (radiatus)	Black gram	T, C, S
Phaseolus vulgaris	Navy bean, kidney bean, pinto bean, French bean, white bean, wax bean, haricot bean, garden bean	T, C, E, S
Pisum sativum	Field bean, garden pea	T
Psophocarpus tetragonolobus	Winged bean, Gao bean	T
Stizobolium deeringanum	Velvet bean	T
Vicia faba	Broad bean, field bean, faba bean	T, C, Th, Pr, Pa
Vigna unguiculata (sinensis)	Cowpea, black-eyed pea, Southern pea, serido pea	T, C
Voandzeia subterranca	Bambara bean	T

[a]C= chymotrypsin; E= elastase; K = Kallikrein; Pa= papain; Pl= plasmin; Pr= pronase; S= subtilisin; T= trupsin; Th= thrombin.
Source: Liener (6), compiled from Liener and Kakade (7).

The physiological effects of proteinase inhibitors differ between animal species (21). Among the sources of variation one may count the extent of the digestion of a diet, the specificities and morphology of the gastro-intestinal tract and the extent of inactivation in the stomach (3). Another important factor that should be taken into consideation is the stoichiometry of inhibitions which may depend on differences in number and potency of secondary binding sites between the inhibitors and the trypsins from different animal species. The inhibitory capacities of BBI, measured in terms of trypsin or chymotrypsin inhibited, are usually evaluated on bovine pancreatic proteinases. This has raised the question of validity and relevance of inhibition data with respect to trypsin and chymotrypsin from other animal species. Indeed, it has been shown that many inhibitors that strongly inhibit bovine trypsin do not inhibit human trypsin (22). STI inhibits more trout trypsin than bovine trypsin (23) and BBI inhibits more carp trypsin and chymotrypsin than the bovine enzymes (24). As for pea proteinase inhibitors, they inhibit more bovine and rat trypsin than porcine trypsin (25). Trypsin and chymotrypsin from rats, mink and pigs showed appreciable differences in their sensitivity toward pea proteinase inhibitors (26). The recently reported differences in the mode of action of lentil proteinase inhibitors against human and bovine chymotrypsin and trypsin have been attributed to a combination of the unsual, additional binding of human chymotrypsin at the trypsin inhibitory site and the weak binding of bovine chymotrypsin (27).

The most striking finding on the effect of proteinase inhibitors is the remarkable enlargement of the pancreas and the increase in pancreatic secretory activity. Ingestion of raw soybean meal or trypsin inhibitors caused pancreatic enlargement in rats, chicks, mice and young guinea pigs but not in adult guinea pigs, dogs, growing swine, claves and presumably, humans (reviewed, 28), and it has not been noted in primates even after 5 years of feeding on soybean-based protein diets containing trypsin inhibitors (29). Pancreata of rats and chicks adapted to raw soybean meal synthesized more trypsinogen and chymotrypsinogen than pancreata of rats adapted to heated soybean meal (30). The trypsin inhibitory site, rather than chymotrypsin inhibitory site of BBI, is involved in the enlargement of the pancreas and in the increase of pancreatic proteolytic activity (31).

The mechanism by which the inhibitor induces the pancreatic enlargement is not yet fully understood. It is explained by a feedback inhibition which depends on the level trypsin present at any given time in the small intestine. When the level of this enzyme falls below a certain critical threshold value, the pancreas is induced to produce more enzyme. The suppression of this negative feddback mechanism can thus occur if the trypsin is complexed with the inhibitor. It is believed that the mediating agent between trypsin and the pancreas is the hormone cholecytokinin (CCK), which is released from the intestinal mucosa when the level of trypsin in the intestine falls below its theshold level (reviewed, 6). The negative feedback mechanism of pancreatic enzyme secretion found in the rat exists also in the pig and calf, which do not develop pancreatic enlargement (28). A recent study has confirmed the existence of feedback control in human (32).

In view of the growing interest in the use of legume seed products in the human diet, it becomes important to assess the effect on human health associated with the consumption of trypsin inhibitors present in a wide variety of foods. In a study carried out in 1977 (33), it has been found that prolonged feeding of male Wistar rats on raw soybean meal enhanced the action of the pancreatic carcinogen azaserine. In the «USDA trypsin inhibitor study», male Wistar rats, which had been fed

raw soybean meal or experimental unheated soy protein isolates for 2 years, developed pancreatic nodular hyperplasia and acinar adenoma in a dose-dependent manner (34). However, similar long-term feeding of mice and hamsters on raw soybean meal, in the presence or absence of chemical carcinogens, failed to induce carcinogenic changes in the pancreata. Moreover, the raw soybean meal seems to have excerted a protective effect on the chemical induction of tumors in the hamster (35). In view of the difference in species response to the presence of inhibitors in the diet, the relevance of the above pancreatic effects in human remains to be elucidated.

Tribolium castaneum, the red flour beetle that causes serious damage to wheat, rice and corn but fails to develop on raw soybeans, served as a model for these studies. The *Tribolium* midgut trypsin-like proteolytic activity was fully inhibited by BBI when co-submitted to polyacrylamide gel electrophoresis in gels embedded with gelatin, but not at all - when assayed on a protein substrate in solution. This points towards the necessity for mutual, «solid state», exposure of enzyme to inhibitor, in a similar manner to the interaction of the insect with solid food (36).

In a series of studies in rats, BBI significantly counteracted the nephrotoxicity induced by the antibiotic amino glycoside gentamicin commonly used in clinical practice. BBI did not show any side effects and did not affect the antimocrobial activity gentamicin (37, 38).

Epidemiological studies have identified legumes as possible protective agents in the decreased occurrence of breast, colon and prostatic cancer in vegetarian population. The association of legume seeds rich in proteinase inhibitors with prevention of human cancers, stimulated the insight into the possible action of proteinase inhibitors as cancer chemopreventive agents. The epidemiologic evidence for a cancer protective role of the inhibitors comes primarily from correlation studies (4, 39) and from evidence based on *in vitro* models rather than on studies in humans. The *in vitro* studies of the anticarcionogenic proteinase inhibitors have recently been compiled by Kennedy (40), showing that BBI proved to be a potent anticarcinogenic agent *in vitro* (41). In experiments on transformation of C3H/10T1/2 cells, it has been shown that nanomolar concentrations of BBI suppress the X-ray-induced transformation *in vitro* and that the chymotrypsin-inhibitory domain of BBI is responsible for this effect (42). The recently isolated, thermostable, trypsin- and chymotrypsin-inhibitor from amaranth seeds effectively blocked estrogen-induced tumorigenesis of MCF7 breast cancer cells *in vitro*. Similar results were obtained with BBI (43,44). Recent studies on the effect of BBI *in vivo* have demostrated the ability of dietary BBI to prevent or suppress carcinogenesis in animal model systems *in vivo* (45, 46).

DISCUSSION

Proteinase inhibitors are a significant component of human food. The evidence that they constitute a hazard to health is frequently only presumptive and must be placed in perspective in relation to the level of total proteinase inhibitors in the overall diet. Most of the *in vivo* research has been done with small animals that consumed large quantities of a particular food component over a long period of time- a situation quite removed from the eating patterns and varied diets of humans. The reported findings point also at remarkable differences between animal species in response to ingested proteinase inhibitors. The *in vivo* differences are supported by the *in vitro* experiments demostrating the variability in inhibitory capacity of a certain trypsin inhibitor towards target trypsins from different animal species. The relevance of *in vitro* assays to conditions prevailing *in vivo*, and different potencies of inhibition achieved under different assay conditions, as found for *Tribolium* trypsin, should also be taken into account. These imply standardization of analytical methods and consequent quantitative evaluation of foods in *in vivo* experiments. Finally, the alleged anti-nutritional properties of legume seed proteinase inhibitors should be weighed against their potential in protecting valuable crops and foods in storage and also as possible anticarcinogens (47).

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Tannins: thermostable pigments which complex dietary proteins and inhibit digestive enzymes

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SUMMARY. The presence of antinutritional factors in legume seeds and other vegetables has been considered as an expression of the chemical warfare of plants against their predators. As a consequence, the nutritional utilization of these foods has only been possible through the use of a variety of treatments (cooking, fermentation, germination) which increase nutrient bioavailability. Nonetheless, some factors are not destroyed by effect of seed processing, among which stand a family of polymeric polyphenols called tannins. These pigments have the ability to complex and precipitate proteins and inhibit digestive enzymes. This paper describes what has been accomplished in regards to the selection of an appropriate solvent to extract bean polyphenols, the assessment of the most commonly used assay procedures, the purification of bean tannins and the evaluation of their interaction with proteins and digestive enzymes, responsible for their antinutritional effect.

RESUMEN. Taninos: Pigmentos termoestables que se acomplejan con proteínas e inhibiben enzimas digestivas. La presencia de factores antinutricionales en las semillas de leguminosas y otros vegetales se ha interpretado como la expresión de mecanismos de defensa de las plantas contra sus depredadores. Por ello, el aprovechamiento nutricional de dichas semillas sólo ha sido posible a través de la aplicación de diversos tratamientos de cocción, fermentación, germinación, etc., los cuales producen, en general, un aumento en la biodisponibilidad de los nutrientes. No obstante, algunos factores resisten a los tratamientos mencionados. Entre estos se encuentra una familia de polifenoles poliméricos, llamados taninos, que se concentratan en la cáscaras de semillas coloreadas. Los taninos tienen la capacidad de acomplejarse con proteínas, inducir su precipitación e inhibir una diversidad de enzimas digestivas. El presente trabajo describe los logros alcanzados en la búsqueda del solvente de extracción más apropiado para solubilizar los polifenoles de semillas de leguminosas, la evaluación de diferentes procedimientos de cuantificación, la purificación de los taninos de las semillas y el estudio de su interacción con proteínas y enzimas responsables de su efecto antinutricional.

INTRODUCTION

Some vegetable foods contain antinutritional and toxic factors which conform an effective arsenal in the chemical warfare of plants against their predators. Legume seeds are an example of a heavy armored group [1]. Nonetheless, the bean's content of proteins, carbohydrates, dietary fiber, minerals and vitamins confers them a high nutritive potential whose extensive exploitation have only been possible through the development of a variety of processing techniques (ordinary cooking, autoclaving, roasting, germination, fermentation, etc.).

Although these treatments had made possible the consumption of pulses, the digestibility of processed seeds is usually lower than that of animal foods. A variety of reasons have been put forward to explain this finding. Besides the intrinsic nature of some seed proteins (*i.e.* globulins) and starches, other determinants contribute to the relatively low digestibility of cooked beans: The incomplete inactivation of heat-labile antinutritional factors (hydrolase inhibitors and lectins), the presence of heat-stable agents (tannins, alkaloyds and non-protein amino acids), and the formation of complexes among various seed components (proteins, starches, lipids, fiber, minerals) which are resistant to hydrolase attack [2,3,4].

Several reports have pointed to the fact that colored seeds had lower digestibilities than the white varieties [5,6]. In addition, rats or chicks fed diets containing colored *Vicia faba* or sorghum grains showed decreased body weight gain. In these animals, the true digestibility of proteins and, to a lesser extent, that of carbohydrates and fats, was sensibly reduced [7,8]. These effects have been attributed to polymeric

polyphenols (tannins) which concentrate in the coats of most colored seeds.

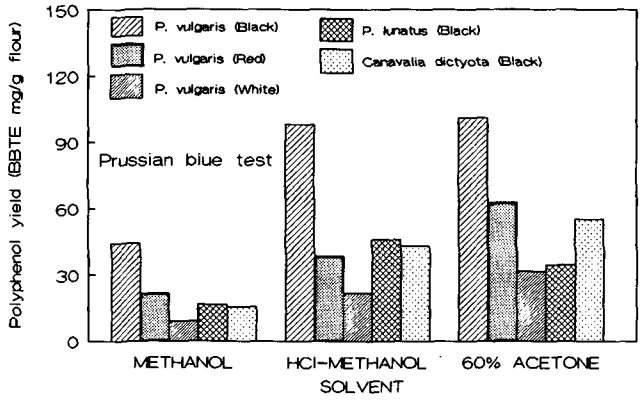
Tannins, an ill defined and chemically diverse class, are able to complex and precipitate proteins [9]. Two major tannin categories have been identified: hydrolyzable and condensed tannins. The latter, abundant in vegetable foods [10], are polymers of flavan-3ols (catechins) or flavan-3,4-diols (leucoantocyanidins). The first group, represented by tannic acid, is composed of esters of phenolic acids with sugars [10,11].

The lack of commercial condensed tannins has prompted the use of tannic acid as a model compound in various **in vivo** and **in vitro** studies depicted to evaluate the antinutritional effects of the whole tannin group. Although some of the changes brought about by the latter are similar to those of condensed tannins, there are also important differences. For instance, diets based on high tannin sorghum do not cause depression of food intake as has been reported for those containing tannic acid [7,12,13]. In addition, condensed tannins seem to be more effective than tannic acid in regards to the reduction of body weight in chicks [14].

To ascertain the antinutritional influence of common bean tannins it was necessary to extract, quantify and extensively purify them, in order to test their effects free of other interferences. These aspects were addressed in the present study.

The ability of methanol, 1% HCl in methanol and 60% acetone to extract legume seed coat polyphenols is depicted in Figure 1. As compared to pure methanol, acidic methanol or the aqueous acetone solution increased two to three times the extraction yield. Water and 60% ethanol were only slightly more effective than methanol (results not shown). Extraction yield was always higher for the colored seeds as compared to white common beans.

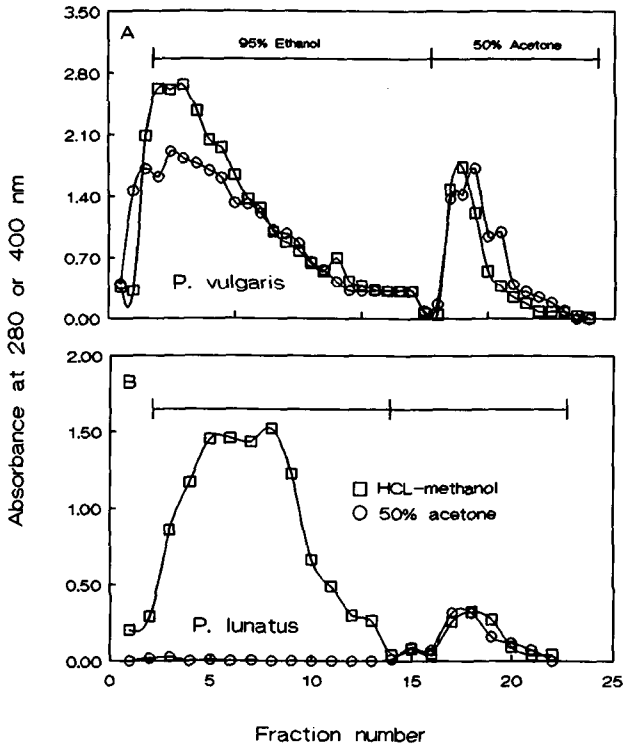
FIGURE 1
Effectivity of various solvents to extract bean polyphenols



Seeds were ground to an average particle size of 820 μm and subjected to four consecutive extractions with the indicated solvents using a flour/solvent ratio of 1:10. Extracts were pooled and their total polyphenol content assessed with the Prussian blue reagent [16]. Results were expressed as Black Bean Tannin Equivalents (BBTE).

The selectivity of extraction solvents to solubilize polyphenols is a frequent concern of researchers in this field. This question can be approached analyzing polyphenol extracts by adsorption chromatography on Sephadex LH-20 [15,16]. In short (4.5 x 2 cm) columns, HCL-methanol and acetone extracts from the coats of *black Phaseolus vulgaris* and *P. lunatus* seeds were resolved into two major fractions: non-tannins eluted with ethanol and condensed tannins eluted with 50% acetone (Figure 2). The chromatographic profiles were very similar for both *P. vulgaris* extracts. For *P. lunatus*, the acidic methanol extract yielded a profile similar to that from *P. vulgaris*, while the acetone one contained almost exclusively condensed tannins. These results indicate that there could be differences in the selectivity of solvents to extract some polyphenols depending upon its source. The uncertainties associated with polyphenol extraction could be solved by direct assessment in the dry vegetable material. Although some attempts have been made using Near Infrared Reflectance Spectroscopy [17] or seed color determination in a chromameter [18], these procedures have not been properly validated.

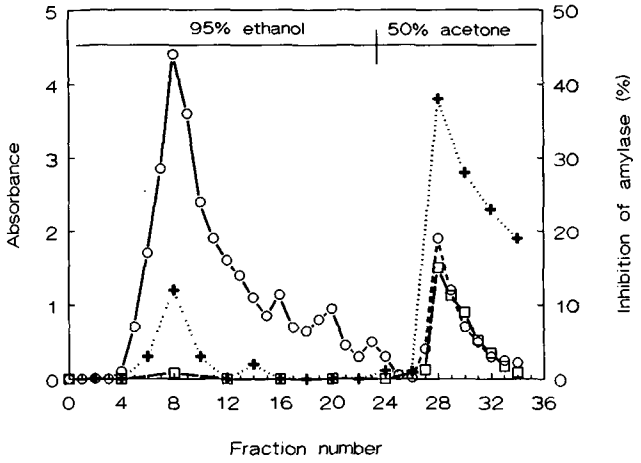
FIGURE 2
Fractionation of *P. vulgaris* and *P. lunatus* polyphenols by adsorption chromatography



HCl-methanol and 60% acetone extracts were seeded on top of a Sephadex LH-20 column (4.5 x 2 cm) [16]. Non- tannin polyphenols were washed with 95% ethanol and their elution followed at 280 nm. Afterwards, tannins were desorbed with 50% acetone and their elution monitored at 400 nm.

The results of a more extensive characterization of common bean polyphenols is presented in Figure 3. Fractions eluted with 50% acetone reacted strongly with the vanillin reagent and inhibited pancreatic α-amylase. These fractions did not react with anthrone (results not shown) indicating the condensed nature of these tannins.

FIGURE 3
Characterization of polyphenols from black beans

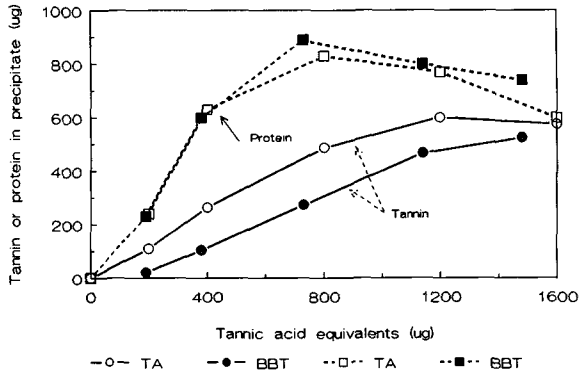


A HCl-methanol extract of black beans was cromatographed in a 20 x 2 cm Sephadex LH-20 column and resolved as indicated in figure 2. Fractions were analyzed for condensed tannins with the vanillin reagent (□) and their ability to inhibit pancreatic α–amylase (+) [16,24].

Tannin-protein interactions are a major issue regarding the nutritional significance of tannins. Using the protein precipitation test designed by Hagerman and Butler [19] it was found that tannic acid and black bean tannins precipitated serum albumin in a similar fashion (Figure 4). Nonetheless, the amount of tannins recovered in the precipitates was usually lower for the condensed ones, particularly at low tannin levels. In spite of the complexities of protein-tannin interactions, these results suggest that bean tannins are more effective promoting albumin precipitation than tannic acid.

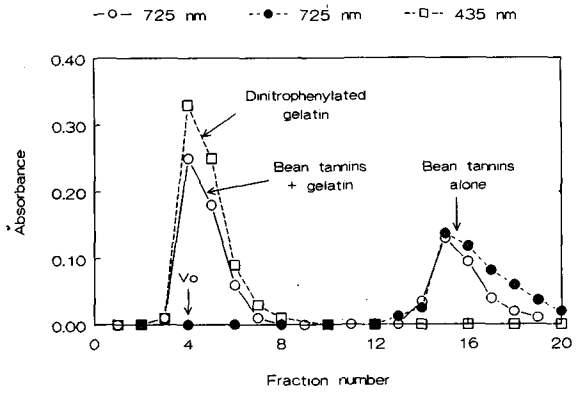
Figure 5 demonstrates the formation of soluble black bean tannins-gelatin complexes. When the supernatant from incubations of gelatin and tannins were chromatographed in Bio-Gel P60, a fraction of the tannins appeared along with the protein in the void volume, while the uncomplexed polyphenols were considerably delayed. These soluble tannin-protein adducts are of utmost importance in regards to enzyme inhibition which may occur at tannin concentration which do not induce protein precipitation.

FIGURE 4
Formation of insoluble tannin-protein complexes



Bovine serum albumin (1 mg/ml) was incubated with the indicated amounts of either black bean tannins or tannic acid [19]. Precipitates were collected, rinsed and analyzed for their tannin content. The amount of protein precipitated was estimated by difference measuring that remaining in the supernatant.

FIGURE 5
Demonstration of soluble tannin-protein complexes



Samples of black bean tannins and of the supernatant from incubations of dinitrophenylated gelatin and black bean tannins were chromatographed on a (0.6 x 29 cm) Bio-Gel P60 column, using 0.15 M NaCl as solvent. The elution of the protein was followed as 435 nm while that of tannins by their reaction with the Folin-Ciocalteus reagent measuring the resulting absorbance at 725 nm. Vo.: Void volume.

Considering that condensed tannins are not absorbed from the gut, the evaluation of their antinutritional potential points to establish their effects on various digestive enzymes: pancreatic proteases (trypsin and chymotrypsin), α-amylase, brush border disaccharidases (maltase, sucrase and lactase) and the intestinal glucose uptake system (Table 1). Black bean tannins inhibited the enzymes concerned with carbohydrate digestion, the proteolytic activity of pancreatin and, to a lesser extent, intestinal glucose uptake. Nonetheless, caution should

be taken in interpreting these figures due to the differences in the assay mixtures employed to measure these enzymes where complex tannin-protein and or tannin-substrate interactions could be established.

TABLE 1
INHIBITION OF DIGESTIVE ENZYMES BY BLACK BEAN TANNINS

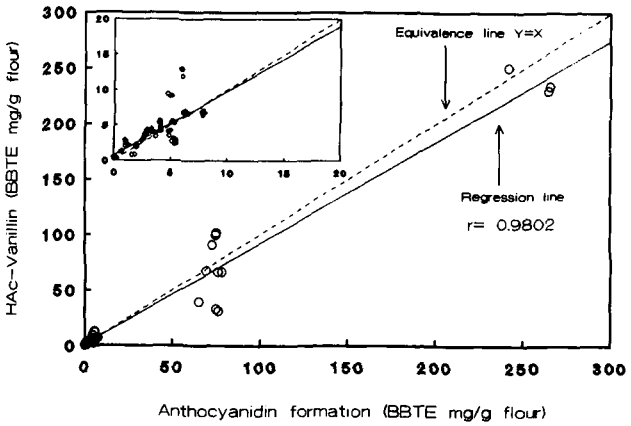
Enzyme[1]	Source	Substrate	Tannin requiered to to inhibit activity by 50% (µg)
Proteases	Bovine pancreatin	Casein	180-200[2]
α-amylase	Bovine pancreatin	Starch	21[3]
α-amylase	Hog pancras (purified)	Starch	50[4]
Maltase	Rat brush border membranes	Maltose	73[5]
Sucrase	Rat brush border membranes	Sucrose	57[5]
Lactase	Rat brush border membranes	Lactose	97[5]
Glucose uptake	Rat *intestinal slevees*	Glucose	> 2000[4]

1. Enzymes were measured using standard assays (21-25).
2. Rojas, M. (unpublished observations).
3. Carmona, A. (unpublished observations).
4. Borges, G. (unpublished observations).
5. Borgudd, L. (unpublished observations).

The concentration dependent nature of the fore mentioned effects of bean tannins emphasize the need to quantify the seed content of polymeric polyphenols able to elicit an antinutritional response. In our laboratory we have critically evaluated a variety of assay procedures, from those based on the general reductive character of polyphenols (*i.e.* Prussian blue test) to those highly specific for condensed tannins such as the vanillin reaction (Figure 2) or the quntitative anthocyanidin formation test [20]. To overcome some of the limitations of the traditional vanillin reaction performed in methanol [16,21], the modified procedure of Butler et al. [22], in glacial acetic acid, proved to be more reliable and led to results which closely paralleled those obtained measuring anthocyanidin release under conditions which minimize phlobaphene formation (Figure 6).

Although the nutritional relevance of the data presented in this study is still uncertain, due in part to the lack of results from *in vivo* experiments, we are opening a way to test the effect of condensed tannins under conditions closer to those prevailing *in vivo*. Considering the consequences of dietary protein complexing and enzyme inhibition, it is likely that condensed tannins may significantly contribute to reduce the digestibility of colored legume seeds.

FIGURE 6
Correlation between the tannin determinations performed with the glacial acetic acid-vanillin and anthocyanidin formation tests



HCl-methanol extracts from the seeds of 8 species and varieties of legumes were analyzed for their tannin content using the acetic acid-vanillin [22] and anthocyanidin formation [20] tests. Results were expressed as Black Bean Tannin Equivalents (BBTE). The equivalence line represents the Y=X theoretical line. The inset expands the scale to show the points around the origin of the plot.

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Bioavailability of carbohydrates in legumes: Digestible and indigestible fractions

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SUMMARY. Despite their important contribution to seed weight, carbohydrates in pulses have received limited attention. However, experimental evidence acumulated during the last two decades indicate that legumes are rich sources of slowly digestible starch promoting moderate postprandial glycemic and insulinemic responses. Although the reasons for this phenomenon are not completely understood, some intrinsic properties of the starch itself and the microstructure of cotyledon cells appear to determine much of the slow release character. This beneficial feature is rather sensitive to thermal and mechanical processing. A minimum of 10% of the starch occurring in common beans and lentils escapes digestion and absorption in the normal small intestine, and is therefore referred to as «resistant starch». This material consists mainly of retrograded amylose fractions generated upon cooling of wet-heated pulses. Physically inaccessible starch fractions resulting from cotyledon microstructural properties may also contribute to incomplete digestibility, accounting for up to 40% of the indigestible starch. These indigestible starch fractions are fermented in the large intestine generating gases and volatile fatty acids, compounds that have important influence on the physiology of the colonic mucosa and peripheral metabolism.

RESUMEN. Biodisponibilidad de los Carbohidratos de las Leguminosas: Fracciones Digeribles e Indigeribles. En contraste con el amplio conocimiento que se tiene del valor nutritivo del componente proteíco de las leguminosas, y pese a su indiscutible relevancia en términos cuantitativos, aún se sabe poco sobre los carbohidratos presentes en estos granos. El componente mayoritario de las semillas maduras de leguminosas es la fracción compleja. Este renglón, que engloba a la fibra dietética y al almidón, puede representar hasta el 60% del peso de la semilla. Una fracción importante del almidón es hidrolizada a una tasa relativamente baja, lo cual se traduce en una respuesta glicemica moderada. Por ello, las leguminosas constituyen un renglón alimentario atractivo para el manejo dietético de la diabetes mellitus. Los mecanismos que determinan este comportamiento incluyen tanto a las propiedades del almidón en sí, como de la microestructura del cotiledón y sus células constituyentes, lo cual lo hace dependiente del procesamiento al que se someten los granos. Otra porción del almidón presente en los granos tiene carácter indigerible. Esta propiedad ha sido caracterizada in vitro e in vivo y parece resultar tanto de la retrogradación de la amilosa, como de la inaccesibilidad física impuesta por la microestructura del cotiledón. La evidencia mas reciente sugiere que al menos el 10% del almidón de las semillas cocidas de P. vulgaris y L. culinaris escapa a la digestión y absorción en el intestino delgado. Así, una porción importante del componente amiláceo de las leguminosas ingresa al intestino grueso donde, junto con los α-galactósidos, es fermentada por la microflora colónica. Este último proceso da origen a los inconvenientes asociados a la generación de gases, pero tiene influencia beneficiosa sobre la fisiología de la mucosa del colon y, posiblemente, sobre el metabolismo lipídico hepático.

Carbohydrates in most edible pulses represent up to 65% of the seed weight. More than a half of this portion consists of the so-called «complex carbohydrates», i.e. starch and dietary fiber (1), while the sugar fraction (mono, di- and oligosaccharides) is significantly smaller (2).

Although from a quantitative point of view carbohydrates are the main constituent of most dried legumes, nutrional studies on pulses have focussed mainly on their proteins. Two major reasons may be argued to explain such a contradiction: *i)* the early perceived idea of food carbohydrates being easily digested and absorbed in the human small intestine, and *ii)* the apparently low impact that these dietary compounds have on health, specially when compared to fat and proteins. Nevertheless, the outcome of numerous investigations during

the last 15 years indicate that the nutritional role of carbohydrates has been largely underestimated, and leguminous foods have provided important evidences for this conceptual change.

In the early 80s, David Jenkins and his research team made an interesting finding when monitoring the evolution of blood glucose in human subjects after the ingestion of experimental breakfasts. Surprisingly, the glycemic responses to legume-based meals were remarkably lower than those registered after other starchy foods or free sugars (3). After these and other experiences, legumes were accepted as sources of «slowly released carbohydrates». The evolution of this pioneer concept has led to a new nutritional classification of carbohydrates (4), which attends to the rate and extent of their digestion in the small intestine, parameters that determine the consequences of carbohydrate ingestion on intestinal and peripheral physiology. Thus, the existence of rapidly digested, slowly digested and indigestible carbohydrates is currently recognized. As it will discussed later, legume seeds contain important quantities of each of these nutritionally relevant carbohydrate types.

The above mentioned «slow release» feature of legume carbohydrates seems to be consequence of the limited susceptibility to enzymatic break down shown by their starches. Compared to cereal and tuber samples, a high proportion of the starch present in lentils and common beans behaves as «slowly digestible starch» *in vitro* (4). This characteristic results in a delayed hydrolysis and absorption of the digestion products *in vivo* promoting, therefore, moderate postprandial hyperglycemia and insulinemia, a fact of potential use in the dietary management of diabetes mellitus (5).

Although the mechanism governing the slow digestion of starch in pulses is not completely understood, it clearly depends on multiple factors. Some of them seem to be intrinsic to the starch itself. This was illustrated for isolated black bean (Phaseolus vulgaris) starch, which is less digestible by pancreatic amylase than wheat, corn or rice starches (Table 1) (6), a fact that might be explained by the relatively high amylose:amylopectin ratio commonly found in leguminous starches (7).

TABLE 1
IN VITRO DIGESTIBILITY OF ISOLATED CEREAL AND BEAN STARCHES[a]

Treatment	Degree of Hydrolysis (%)			
	Black Bean Starch		Corn Starch	
	15 min	60 min	15 min	60 min
None	5	10	3	8
Boiling (30 min)	30	50	50	75

[a]Modified from Socorro, Levy-Benshimol & Tovar (6)

Microstructure is, however, a major determinant of the digestion rate of starch in legumes. The remarkable mechanical resistance of common bean cotyledon cell walls was demonstrated by Würsh et al. (8), who found that gentle mashing of boiled white beans yields a paste rich in rather intact cotyledon cells containing starch granules. Such a unique physical property allowed us to process common beans and lentils into slowly digestible precooked flours (9,10), in which the occurrence of cell wall-surrounded starch was noticeable (11). The influence of cell wall integrity on the *in vitro* starch hydrolysis of the processed powders was made evident by the substantial increase in the amylolysis rate recorded after exhaustive homogenization (Table 2). Recent studies indicate that this phenomenon may also limit protein digestibility in cooked beans (12).

TABLE 2
EFFECT OF CELL WALL DISRUPTION ON *IN VITRO* DIGESTIBILITY OF STARCH IN PRECOOKED LEGUME FLOURS[a]

Treatment	Degree of Starch Hydrolysis (%)			
	Red Beans		Lentils	
	15 min	60 min	15 min	60 min
None (control)	8	19	11	31
Homogenization	50	54	55	73

[a]Modified from Tovar et al. (11)

In vivo trials have confirmed that microstructural alterations induced by processing modify metabolic responses to starch in legumes (13). Table 3 summarizes the glycemic and insulinemic indices of variously treated red kidney beans. The cooking procedure has an evident impact on both glycemic and insulinemic responses to the seeds, since autoclaved samples have greater indices than boiled ones, suggesting that pressure cooking might produce a more extensive damage to cell wall structures. Further deterioration of the seed structural integrity, achieved by milling during the preparation of precooked flours, resulted in increased metabolic responses (Table 3). Therefore, the architecture of the seed tissues appears to constitute a physical barrier that limits digestive enzymes action on their starch substrate. Higher levels of structural damage, such as cell wall disruption by homogenization of the precooked flours, yielded more rapidly digested preparations with greater metabolic indices.

TABLE 3
GLYCEMIC AND INSULINEMIC INDICES OF VARIOUSLY TREATED RED KIDNEY BEANS [a]

Preparation	Glycemic Index	Insulinemic Index
Boiled Beans[b]	44	34
Autoclaved Beans[c]	58	51
Precooked Bean Flour	62	52
Precooked Flour (without cells)	76	51
Wheat Bread (reference)	100	100

[a]Modified from Tovar et al. (13)
[b]70 min
[c]121 °C, 20 min

It should be stressed that even if postprandial blood responses increase, not all the beneficial 'slow' features are lost during processing, as the greatest glycemic/insulinemic indices recorded for the legume-based breakfasts are still smaller than for the reference cereal sample (bread) (Table 3). This is also valid for the long-lasting satiating effect of red bean meals (Table 4), which correlates with the pace of starch processing in the small intestine (2).

TABLE 4
SATIETY SCORE OF RED BEAN AND WHEAT BREAD MEALS[a]

Postprandial Time (min)	Red Bean Autoclaved	Red Bean Precooked Flour (Without Cells)	Wheat Bread
30	5.6	6.3	3.3
95	3.4	2.5	-0.3
120	2.3	0.7	-1.0
180	0.5	-1.8	-3.9

[a] Modified from Tovar (2)

The rate of starch digestion in freeze-dried samples from cooked beans appears to be different from that of the material leaching out to the cooking water (Tovar J, unpublished). In spite of the presence of cell-enclosed starch in both fractions, the digestibility of the cooking outflow was significantly lower. Although it is tempting to especulate about differential deterioration of cellular structures in each preparation, different starch dispersion properties may also account for unequal susceptibilities to amylolysis. This finding deserves further attention, particularly in view of the traditional latinamerican use of bean cooking water for infant feeding. In this context, slow -and perhaps incomplete- digestion of ingested starch might be undesirable rather than beneficial.

In vitro studies carried out at this laboratory suggest that, on top of the structural influence of cotyledon cell walls on starch bioavailability, polymeric constituents of legume dietary fiber may interfere with α-amylolysis. The phenomenon is observed even when the fiber is present in a less organized physical state, such as in the indigestible residue isolated by enzymatic procedures. For instance, black bean starch degradation is atenuated by the indigestible residue obtained from the same seeds (Table 5) (6). Among possible reasons for this inhibitory effect of bean fiber, enzyme insolubilization and substrate masking (14), as well as anti-amylase activity of fiber-associated tannins (14,15) have been proposed. It is important to mention, however, that the physiological relevance of these observations is yet unclear.

TABLE 5
EFFECT OF BLACK BEAN INDIGESTIBLE RESIDUE ON *IN VITRO* HYDROLYSIS OF ISOLATED BEAN STARCH[a]

Treatment	Degree of Hydrolysis[b] (%) 15 min	30 min	60 min
None (control)	28	43	45
+ Indigestible Residue	17	24	32

[a]Modified from Socorro, Levy-Benshimol & Tovar (6)
[b]Porcine pancreatic amylase

Indigestible starch fractions occur in most starchy foods (4,16), but legumes are among the richest sources of such a novel dietary constituent. As a matter of fact, at least 10% of the starch in processed legume seeds escapes digestion and absorption in the small intestine and is, therefore, regarded as «enzyme resistant» or, more simply, «resistant starch» (16,17). Accurate assessment of the resistant starch content in these seeds poses the same technical problems faced with practically

any other edible vegetable (17). Thus, we currently rely on estimates obtained with a number of different experimental approaches.

Attempts to measure «true digestibility» of starch in processed common beans gave values ranking from 80 (18) to 90% (19), depending on the *in vivo* model employed. Similar figures have been reported for heat treated lentils (19) and cowpeas (20) (Table 6). These values correspond to a 10-20% resistant starch content, expressed on a total starch basis. In addition to the experimental model, differences in sample origin, agronomical variety and processing conditions may explain the observed discrepant results. Interestingly, and regardless of the digestibility index recorded, undigested starch seems to consist of two major fractions. The predominant one is likely to be retrograded amylose (18-20), a crystalline and enzyme-resistant form that is produced after cooling of heat treated starch or starchy foods (4,16). This observation is consistent with the known tendency of legume starches to generate *in vitro* indigestible retrograded fractions (9,10,16,22).

TABLE 6
IN VIVO DIGESTIBILITY OF STARCH IN PROCESSED LEGUMES

Sample	Method of Assessment[a]	Digestibility Index (%)[b]	Reference
White Beans	HI	80	Schweizer (18)
White Beans	AR	88	Asp et al. (21)
Red Beans	AR	90-92	Tovar et al. (19)
Cowpeas	IC	90	Tuan,Phillips (20)
Lentils	AR	89	Tovar et al. (19)

[a]HI: Human ileostomists; AR: Antibiotic-treated rats; IC: Analysis of ileal content in rats
[b]Undigested starch measured (g) x 100)/starch intake (g)

The remaining of the *in vivo* indigestible starch - approximately 30 to 40% of the resistant starch in beans and lentils- has been poorly characterized, although it is thought to be the sum of part of the physically inaccessible (cell-enclosed) starch mentioned above (19) and the recently acknowledged losses due to physiological inefficiency, an apparently general feature of normal starch assimilation (23).

The occurence of significant amounts of indigestible starch in pulses raises two major questions: should this starch be added to the dietary fiber content of the food? and, is it lost from the physiological point of view? No conclusive answer can be given for the first query which is subject of controversy when the composition of processed foods is to be considered (16). Nonetheless, there is no doubt about the contribution of *in vitro* resistant starch to the dietary fiber residue obtained by enzymatic means (Table 7).

TABLE 7
STARCH CONTRIBUTION TO THE INDIGESTIBLE RESIDUE IN RED KIDNEY BEANS[ab]

Flour	Indigestible Residue[c]	Starch[d]	Dietary Fiber[e]
Raw	18.9	-	18.9
Precooked	22.8	3.5	19.3

[a]Modified from Tovar et al (9)
[b]Values are expressed in g per100 g original flour (dry weight basis)
[c]Obtained after sequential digestion with Termamyl^{R}, pepsin and pancreatin
[d]Assessed enzymatically in the indigestible residue after alkaline dispersion
[e](indigestible residue - starch)

Regarding the physiological use of legume resistant starch, it has proven to be fermented to a variable extent in the large intestine. Indirect estimates suggest that bean indigestible starch is more easily degraded in the rat caecum than the corresponding lentil fraction. Similarly, *in vitro* studies with rat intestinal microflora indicated important differences between resistant starch isolated from these two legume species (24). Hence, indigestible starch adds to the well known legume indigestible oligosaccharides (25) as substrates for bacterial fermentation, a process that generates gases (hydrogen, carbon dioxide and methane) with concomitant undesirable effects. Fermentation, however, also results in volatile fatty acid production (acetate, propionate and butyrate), compounds that contribute to metabolizable energy (26) and seem to exert beneficial action on the colonic mucosa physiology and hepatic metabolism (27,28).

Further research on chemical and nutritional features of legume carbohydrates may help technological manipulation aiming to retain, or even increase, the amount of slowly and indigestible fractions in pulse-based foods for affluent societies. Research for developing countries should be, in contrast, focussed on the improvement of the overall digestibility.

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Mejoramiento genético de leguminosas

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RESUMEN. El mejoramiento genético, comprendido como el proceso que permite obtener poblaciones con alta frecuencia de fenotipos deseables, incluye métodos llamados tradicionales y biotecnológicos. Mediante este proceso se puede resolver cualquier problema, con la única condición de que esté asociado a una característica que sea controlada por genes. Por esta vía, en las leguminosas se han mejorado el rendimiento, la morfología, el comportamiento ante patógenos y otros factores del ambiente que causan estrés, y la calidad de los granos. En Venezuela, un ejemplo importante de lo que puede hacer el mejoramiento genético lo constituye la domesticación de la especie *Canavalia ensiformis* (L.) DC. y la solución de algunos problemas que limitan su cultivo, entre ellos se destacan el porte o hábito de crecimiento (se pasó de una planta erecto-voluble a una más erecta) y los frutos que se pudren por el contacto con el suelo (se han logrado plantas con frutos más cortos). Para el problema de los factores antinutricionales también se pueden alcanzar soluciones; se han encontrado genotipos con menores contenidos del aminoácido libre canavanina.

SUMMARY. Genetic improvement of legumes. Genetic improvement, a process aimed to obtain populations with a high frequency of desirable phenotypes, comprises both traditional and biotechnology approaches. Through these manipulations it is possible to address any problem, provided it is associated with a gene determined character. In the case of legume crops, some features have been changed such as yield, plant morphology, susceptibility to pathogens and other stress factors which reduce grain quality and output. In Venezuela an example of genetic improvement is represented by the domestication of *Canavalia ensiformis* (L) DC. working out some problems which limited its use as a crop, such as the shape and growing pattern, to obtain erect plants, and pod length to avoid its contact with the humid soil which accelerates rotting. Regarding the improvement of grain quality, genotypes have been found with low canavanine content.

El mejoramiento genético de las leguminosas, al igual que en todas las especies vegetales, ha sido desarrollado científicamente durante el siglo XX, a partir del redescubrimiento de las Leyes de Mendel. Esta ciencia y arte se puede definir como el proceso que permite obtener poblaciones con alta frecuencia de fenotipos deseables. La palabra fenotipo que se incluye en esta definición implica, de una vez, que a los genotipos mejorados se les debe ofrecer el ambiente adecuado para que manifiesten la característica mejorada. Por otra parte, el proceso puede incluir métodos tradicionales, como introducción y evaluación, selección con y sin recombinación, hibridación e inducción de mutaciones, o los más novedosos conocidos como biotecnológicos (cultivo *in vitro* e ingeniería genética).

Aunque todos los métodos conducen a los tres tipos de cultivares que se utilizan en la producción comercial de las especies vegetales, el clón (un genotipo multiplicado vegetativamente), la variedad (grupo de genotipos reproducidos por autofecundación) y el híbrido (un genotipo obtenido por cruzamiento entre dos líneas puras). Para iniciar un programa de mejoramiento se requiere conocer la forma de reproducción de la especie.

En su forma tradicional el mejoramiento genético se aplica siguiendo las etapas siguientes:

1. Búsqueda de variabilidad genética
2. Determinación de la naturaleza de esa variabilidad.
3. Aplicación de un método apropiado.
4. Evaluación y comparación de los genotipos obtenidos.

Después de demostrar sus bondades, el punto final debe ser la entrega del cultivar obtenido a los agricultores. La aceptación o no de ese genotipo ya no es responsabilidad del mejorador.

Los métodos nuevos permiten ahorrar tiempo (generaciones), por ejemplo: para obtener individuos homocigotas, se cultivan partes haploides como el grano de polen y se le duplican los cromosomas; y superar barreras que, para los procedimientos tradicionales, serían insalvables; por ejemplo: para usar genes de una especie a otra se puede emplear desde la fusión de núcleos hasta la transformación.

En general, a través del mejoramiento genético se puede resolver cualquier problema; la única limitante es el posible control genético. Una medida del control genético es la heredabilidad, la cual es un cociente entre la varianza debida a la diferencia entre genotipos y la varianza debida a la diferencia entre fenotipos (la suma de la causada por los genotipos más la causada por el ambiente). Sin embargo, esa limitante depende de la forma como se defina el problema; en este sentido, puede suceder que al descomponerlo, alguna de las características componentes tenga una heredabilidad alta. También se pueden buscar características que sin ser componentes del problema tengan alguna asociación con él. Por ejemplo: el rendimiento de granos es uno de los objetivos más comunes del mejoramiento. Una descomposición usual del rendimiento señala que este es la resultante del producto de tres características: número de frutos por planta, número de semillas por fruto y peso de semilla. La importancia de cada característica puede depender de la especie; así: en arveja, el número de frutos es la más importante(1); mientras que en soya de crecimiento indeterminado el número de semillas parece más importante(2).

Un problema comúnmente considerado en la leguminosas es su morfología, entre las características consideradas están el hábito de crecimiento; en esta familia es frecuente encontrar, dentro de una misma especie, diferentes hábitos tales como plantas rastreras, trepadora y arbustos («maticas»). Estas últimas son las más convenientes para la producción comercial porque se adaptan mejor a la mecanización de las labores. Esta característica está estrechamente asociada con el patrón de crecimiento, determinado e indeterminado; en general, hay mayor preferencia por el determinado. Tanto el hábito como el patrón tienen heredabilidades relativamente altas (3).

Las leguminosas son plantas muy atractivas para los enemigos naturales ya sean insectos, hongos, bacterias o virus. Por otra parte, el mejoramiento genético ofrece la solución más definitiva para ese tipo de problema. De allí que éste sea otro de los problemas más estudiados en esta familia.

La calidad de los granos es uno de los problemas que más se estudia actualmente. En los granos de estas plantas se pueden acumular altas cantidades de los compuestos nutritivos básicos, esto es: proteínas, carbohidratos y lípidos. En muchos programas se ha buscado aumentar los contenidos de estas sustancias; sin embargo, en algunos casos se ha encontrado que el incremento de una trae como consecuencia la disminución de otra; tal situación se ha presentado por ejemplo en soya, en la cual el contenido de proteína podía estar inversamente correlacionado con el de aceite (3). No obstante, en relación a las proteínas se han logrado detalles importantes como cultivares de soya con alto contenido de aminoácidos azufrados (4). Por otra parte, la acumulación de altas cantidades de nitrógeno en estas plantas puede conducir a la formación de metabolitos secundarios que pueden, a su vez, producir efectos indeseables en los animales tales como disminución de la cantidad de alimento consumido. En los animales utilizados en explotaciones pecuarias, este efecto puede limitar los procesos productivos tales como el engorde, la postura de huevos y la producción de leche. A pesar de que existen tecnologías que pueden reducir o desnaturalizar estos metabolitos, el mejoramiento genético sigue siendo la mejor alternativa para modificarlos.

Aunque las leguminosas son plantas que pueden crecer en ambientes muy diversos, por ejemplo: bajo sequía, el estrés causado por diferentes factores del ambiente, como la acidez del suelo y la misma sequía, se está considerando en muchos programas de mejoramiento. Un buen ejemplo de adaptación a un factor del ambiente son los cultivares de soya desarrollados para los diferentes fotoperíodos de EUA y Canadá (3).

De todas maneras, el rendimiento sigue recibiendo atención y mucho del trabajo que se realiza en los otros problemas mencionados van dirigidos hacia su incremento. Así, se puede destacar que las modificaciones morfológicas pueden permitir una arquitectura de la planta más adecuada para la mejor captación de la radiación solar. Otro aspecto relacionado con el rendimiento es su estabilidad, ya no es suficiente con obtener cultivares rendidores, sino que debe buscarse que ese rendimiento pueda mantenerse en años sucesivos y, si es posible, en localidades diferentes.

Dos características en las que se han estado trabajando en los últimos años son el mejoramiento de la capacidad de fijación de nitrógeno y la capacidad de regeneración de plantas en cultivo de tejidos.

Una leguminosa que ha recibido especial atención en Venezuela y otros países es la *Canavalia ensoformis* (L) DC. Las razones de esta atención son, entre otras, su alta capacidad de producción de granos en condiciones tropicales y el alto contenido de proteínas y carbohidratos de sus granos. Sin embargo, esta planta se encontraba en una condición de semisilvestre o semicultivada. Un rasgo de planta cultivada lo constituye el tamaño grande y la alta capacidad germinativa de sus semillas, mientras que el crecimiento indeterminado y la alta dependencia de la humedad ambiental de su reproducción la señalan como silvestre. Muchas de las modificaciones que se han logrado en canavalia han ayudado a su domesticación.

Una primera modificación fue la del hábito de crecimiento o porte erecto-voluble. A esta característica se le considera indeseable porque constituye un impedimento para la realización de labores como la eliminación de malezas y recolección de frutos ya que las plantas se unen unas a otras a través de los llamados ápices volubles. Además de la presencia de estos ápices, este problema tiene dos componentes más la ramifica-

ción excesiva y la caída (decumbencia) de las ramas. Estos dos componentes tienen relación con otros dos problemas, la ramificación excesiva con la desuniformidad de la maduración de los frutos y la decumbencia de las ramas con el contacto de los frutos con el suelo. De los tres componentes el que ha resultado más fácil de mejorar ha sido la presencia de ápices volubles. Con ese propósito, en una población de plantas del genotipo «Original» se diferenciaron dos grupos, uno con alta frecuencia de plantas con ápices volubles y otro sin esos ápices. Luego de dos generaciones, estos grupos se estabilizaron y mantuvieron la diferencia y constituyeron los genotipos, «Postrado» y «Erecto», respectivamente (5). Aunque la presencia de estos ápices depende mucho de factores del ambiente (la sombra, por ejemplo), ha demostrado también una dependencia genética importante.

Una segunda modificación importante ha sido la reducción del tamaño de los frutos. La necesidad de esta modificación surge del problema que constituye el contacto de los frutos con el suelo el cual ocasiona, a su vez, dos dificultades, la pudrición de los frutos en ambientes húmedos y el comienzo de la germinación de las semillas dentro del fruto. Este problema tiene, aparte del que se mencionó (la decumbencia de las ramas), otros dos componentes importantes que son el gran tamaño de los frutos y su inserción muy baja en la planta. Cada componente se ha tratado de mejorar, sin embargo, debido a la herencia relativamente sencilla, dos pares de genes (6), el tamaño del fruto ha sido el que ha ofrecido mayor perspectiva. No obstante, la reducción de los frutos tiene un riesgo muy alto porque se ha demostrado que el alto rendimiento de esta planta depende del tamaño de sus frutos (7). En este sentido, se ha optado por un tamaño intermedio que, en ensayos preliminares, ha permitido obtener genotipos con rendimiento similares a los de frutos grandes.

De los factores antinutricionales que contienen los granos de canavalia, el que parece más superfluo es el aminoácido libre canavanina. A él se le ha atribuido un papel protector de la planta debido a su efecto insecticida (8), pero la comparación, en diferentes ambientes, entre un genotipo de bajo contenido, como «Tovar», con otros de alto contenido no ha comprobado ese papel. Por otra parte, su efecto deprimente sobre el consumo de alimentos en pollitos de engorde si ha sido demostrado (9) y, en consecuencia, el contenido de proteína cruda de 31% en los granos ahora se corrige a 26% (10). Un primer intento para determinar el control genético del contenido de canavanina ha indicado que se debe a pocos pares de genes (posiblemente dos o tres), esto ha permitido que en sólo tres generaciones se puedan obtener genotipos de contenido relativamente bajo.

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Importancia agronómica y nutricional de las leguminosas

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RESUMEN. La distinción ecofisiológica, agronómica y económica de las leguminosas la constituyen los tubérculos y nódulos de su sistema radical; la nodulación ocurre en la gran mayoría de las leguminosas para lo cual es condición que las Rhizobias compatibles estén presentes en la rizófera. Este hecho, las independiza en alto grado del abono nitrogenado, el más costoso de los insumos de la agricultura cerealera moderna. También enriquecen el suelo con nitrógeno cuando se practica la rotación cultural o cuando se utilizan como abonos verdes. La incorporación masiva de las leguminosas en los procesos productivos agrícolas constituye un prerrequisito para el éxito de una estrategia agrícola. Los atributos antes mencionados, aunados a la masificación del uso de subproducto, coproductos y resíduos agrícolas, incluyendo las excretas, permite sustituir en alto grado los fertilizantes químicos, especialmente los nitrogenados. En las zonas y áreas de clima templado se utilizan masivamente las leguminosas, las cuales además de ser base fundamental de la producción animal, aportan al año 18 kg de leguminosas de grano y 60 kg de soya. Tal utilización es muy pobre en los países del Tercer Mundo, ubicados en su mayoría en áreas tropicales; en ellos, sólo se producen anualmente 9 y 12 kg de granos leguminosos y de soya, respectivamente, destinándose una elevada proporción de la soya producida a la exportación. Se propone una ingestión diaria promedio y 70 g de proteína, similares a las que prevalecían en los países desarrollados hace 40 años. Debe propenderse a una disponibilidad de 60 g de leguminosas de grano/persona/año, equivalente a 21,9 kg/persona/año. Se debe avanzar rápidamente hacia la utilización masiva de las leguminosas y, al mismo tiempo, fortalecer la investigación sobre especies de esta extraordinaria familia botánica, mediante la conformación de un Grupo Interdisciplinario para el Estudio e Investigación en Leguminosas (GIESIL).

SUMMARY. Agronomic and nutritional importance of legumes. The main ecophysiologic, agronomic and economic feature of legume plants is the development of tubercles and nodules in their apical system. Nodule formation occurs in most legume species provided a compatible type of *Rhizobium* bacteria is present in the soil. Nitrogen fixation in nodules renders these plants independent of nitrogen fertilizers, the most expensive of all goods in modern cereal agriculture. Considering that soils may get enriched in nitrogen through fixation in nodules and the decomposition of foliage when the aerial parts of legume plants are used as green fertilizers, only through the inclusion of legume crops within planned harvest schemes, it would be possible to achieve success in large scale production strategies. Legume crops are extensively produced in temperate climates areas in which, in addition to their use in animal nutrition, yields of 18 kg per person per year are obtained. In contrast, in the Third World countries located in tropical areas, legume production is scarce, with annual yields of 9 kg per person per year. Currently, it is proposed that the energy and protein intake should match that of the developed countries 40 years ago (*i.e.* 3000 Kcal and 70 g protein per day); for this, it would be necessary to have an average availability of 60 g of legume seeds per person per day. Therefore, the production of legume seeds should be increased. In addition, research aimed to study and exploit the agronomic potential of this rich botanical familiy should be strenghened through the formation of interdisciplinary groups.

Esta familia botánica que incluye 184 géneros y 19.700 especies, es superada sólo por las familias de las orquídeas y las compuestas, y en importancia económica, por las gramíneas.

La familia, que ofrece una de las más extraordinarias riquezas de la biodiversidad del trópico latinoamericano, incluye grandes árboles, arbustos, enredaderas leñosas y herbáceas, hierbas anuales y perennes. En particular, muchas especies de las subfamilias Mimosoideas y Cesalpinoideas son muy apreciadas como árboles madereros, de sombra, ornamentales y fuentes de colorantes, taninos, resinas, gomas insecticidas y medicinas. Por otra parte, numerosos miembros de la subfamilia Papilionoideas son de gran importancia económica como fuente de alimentos de alto valor nutritivo para consumo humano y animal, abonos verdes, cultivos de cobertura y su flores como uno de los mejores recursos para las abejas productoras de miel.

La distinción ecofisiológica, agronómica y económica de las leguminosas la constituyen los tubérculos y nódulos de su sistema radical, la nodulación ocurre en la gran mayoría de las leguminosas, para lo cual es condición que las Rihizobias compatibles estén presentes en la rizófera. Este hecho las independiza en alto grado del abono nitrogenado, el más costoso de los insumos de la agricultura cerealera moderna. También enriquecen el suelo con nitrógeno, cuando se practica la rotación cultural o cuando las leguminosas se utilizan como abono verde. Por lo anterior, la incorporación de las leguminosas en los procesos productivos constituye un prerrequisito para el éxito de una estrategia agrícola.

Puede afirmarse que la alimentación del hombre y la de los animales, particularmente de las especies de explotación económica, es un proceso complejo, en el sentido de que la carencia (o marcada deficiencia) de cualquiera de los nutrientes y/o una limitación en la provisión de energía o un imbalance entre nutrientes o, entre éstos y el nivel calórico de la ración, puede ocasionar serios transtornos, y en el caso de los animales de explotación económica, afectar el proceso productivo, o al menos deteriorar la economía de la explotación. Sin embargo, aunque se acepte el carácter fundamental y complementario de todos los nutrientes, hay que reconocer la trascendencia del nitrógeno como elemento primordial indispensable para la síntesis de los aminoácidos, a partir de los cuales los organismos vivos construyen sus propias proteínas, tanto estructurales como funcionales.

El nitrógeno constituye más de las tres cuartas partes del aire, del cual se extraen muchos millones de toneladas de N anualmente y cantidades proporcionales regresan al mismo como consecuencia de las combustiones y por la descomposición de los residuos vegetales y animales, dentro del llamado **Ciclo del Nitrógeno**. Sin embargo, a pesar de su abundancia, este elemento no puede ser utilizado directamente por las plantas y animales. Su utilización implica que debe ser trasladado desde el suelo, a través de raíces, para, eventualmente, integrarse al metabolismo vegetal. Existen varios procesos naturales y artificiales que permiten suplir de nitrógeno al suelo. Entre estos se incluyen: 1. Formación de óxido de nitrógeno en las tormentas; 2. Fijación biológica de nitrógeno por las algas azul-verde; 3: Fijación biológica por las bacterias libres del suelo; 4. fijación biológica, mediante asociación simbiótica, de las bacterias del género Rhizobium (y también de otros géneros) y plantas de la familia de las leguminosas (y probablemente de otras familias).

A esos cuatro procesos naturales ya señalados debe agregarse el reciclaje que ocurre mediante la descomposición e incorporación al suelo de productos, subproductos, coproductos y desperdicios vegetales y animales.

Como lo refiere Hutton (1), las cantidades de nitrógeno suplidas por las tormentas, las algas azul-verde y las bacterias libres del suelo son relativamente bajas, exceptuando a algunos cultivos de inundación como el arroz donde las algas azul-verde, que crecen en la superficie del agua de inundación, pueden fijar importantes cantidades de este elemento. Por otra parte, los avances tecnológicos agrícolas hacen que cada vez sean menores los restos de cosecha que se incorporan al suelo y el uso de las deyecciones humanas y animales, con frecuencia se ve dificultado por la ubicación urbana de grandes núcleos poblacionales y, en todo caso por la carencia de infraestructuras que permitan transportarlos hacia zonas agrícolas. Lo anterior convierte a la fijación de N en los nódulos de las leguminosas en el proceso más importante y útil que permite lograr, no solamente alimentos de alta concentración proteica para consumo humano sino también enriquecimiento nitrogenado del suelo, para otros cultivos.

El nitrógeno puede ser también artificialmente incorporado al suelo mediante la fertilización química con úrea (46% de N), nitrato de amonio (35% de N) y sulfato de amonio (21% de N). Sin embargo, la síntesis industrial de estos compuestos es costosa. En este sentido, la incorporación racional y diversa de las leguminosas en los procesos productivos agrícolas es quizás la vía más eficiente para propender a la independencia de los fertilizantes químicos nitrogenados.

De lo antes expuesto pudiera concluirse que existen tres vías principales, no excluyentes, para optimizar dentro de una agricultura racional, el aporte de nitrógeno al suelo y, de esta manera, asegurar el suministro adecuado de proteína para consumo directo de la población humana y para la alimentación de los animales domésticos. Tales vías son: 1. Síntesis de fertilizantes nitrogenados. 2. Racionalización del manejo, procesamiento y utilización de subproductos, coproductos y residuos agrícolas, particularmente las deyecciones humanas y animales e incorporación de los llamados abonos verdes. 3. Utilización masiva de las leguminosas en la agricultura, tanto como cultivos de rotación, asociación o cultivos individuales.

Es muy desigual el uso de las leguminosas en los países de clima templado y en los países tropicales. En los primeros las cultivan y producen con alta eficiencia, mientras que en los tropicales, aunque las leguminosas de grano son básicas en la conformación del componente proteico de la dieta de la población, su rendimiento es pobre y prácticamente ausente el cultivo de las leguminosas oleaginosas y forrajeras.

La Tabla 1 muestra la evolución en las últimas décadas de la superficie cosechada, en rendimiento y producción de las leguminosas de grano tradicionalmente consumidas directamente por el hombre y de la soya. Para 1992 los países desarrollados producían por habitante, aproximadamente 18.6 kg de leguminosas de grano y 50 kg de soya, mientras en los países del Tercer Mundo, tal producción alcanza a 9 y 13 kg, respectivamente, estos últimos exportan al Primer Mundo más de la mitad de la soya que producen. En Colombia y Venezuela, países en los cuales se producían 9 y 11 kg de leguminosas de grano por habitante en 1955, reducen tal producción a 3.8 y 3.4 kg respectivamente en 1990.

TABLA 1

	LEGUMINOSAS DE GRANO Consumo Directo					SOYA			
	Superficie cosechada (1000 ha)								
	1948-50	**1965**	**1975**	**1990**		**1945-52**	**1965**	**1975**	**1990**
Mundo	53812	67897	62968	68887	Mundo	15058	25764	38795	56339
P. Desarrollados	10369	14871	10347	12413	P. Desarrollados	5501	15110	23017	25469
Tercer Mundo	43173	53025	52621	56074	Tercer Mundo	9558	10653	15777	30870
Colombia	153	172	220	262	Colombia	4	30	88	116
Venezuela	117	102	101	112	Venezuela				5
	Rendimiento (kg/ha)								
Mundo	513	669	636	863	Mundo	943	1230	1657	1913
P. Desarrollados	607	802	928	1758	P. Desarrollados	1356	1582	1907	2248
Tercer Mundo	489	542	578	666	Tercer Mundo	708	730	1293	1636
Colombia	466	523	637	704	Colombia	1250	1684	1924	1999
Venezuela	487	432	416	581	Venezuela				1800
	Producción (1000 t)								
Mundo	27569	45397	40033	59430	Mundo	14244	31677	64278	107767
P. Desarrollados	6461	11932	9605	21824	P. Desarrollados	7416	23902	43885	57260
Tercer Mundo	21108	33467	30428	37606	Tercer Mundo	6783	7775	20393	50507
Colombia	72	90	140	122	Colombia	5	50	169	232
Venezuela	56	44	42	65	Venezuela				9

Fuente: (2,3)

El cultivo de las leguminosas forrajeras que, con los cereales y la soya, constituye la base de las producciones animales con rumiantes en los climas templados, es también muy pobre en los trópicos, si se exceptúa su importante utilización en Australia tropical. Con respecto a Venezuela, Chacón (4), afirma: «En las diferentes regiones ecológicas de Venezuela, se encuentra una gran diversidad de leguminosas adaptadas a amplias condiciones edáficas y aún cuando representan un gran potencial para la producción animal, no se les ha prestado la debida atención. Este recurso forrajero constituye quizás la alternativa de mayor importancia para mejorar la calidad de la dieta de los animales de pastoreo».

En el trópico está extendido el uso del Kudzú tropical (*Pueraria phaseoloides*) como cultivo de cobertura en las plantaciones de palma africana. Sin duda, las tendencias de las leguminosas de grano y forrajeras en América Latina, contribuyen al cuadro nutricional nada promisorio que impera en la región, porque si bien se logró pasar de las 2353 calorías y 62 g de proteína/persona/día, como disponibilidad promedio en 1961-63 a las 2693 calorías y 66.9 g de proteína/persona/día en 1979-81, no se han logrado nuevos avances hasta el presente. Particularmente trágico resulta el caso venezolano al reducirse la disponibilidad promedio/persona/día de 2719 calorías y 68.9 g de proteína en 1979-81 a 2443 calorías y 61 g de proteína en 1988-90 (3). Para 1993 según evaluación de la División de Nutrición en Salud Pública del Instituto Nacional de Nutrición, la disponibilidad promedio diaria se ubicó en 1932 calorías; una reducción similar debe haber ocurrido para la disponibilidad proteica, lo cual la ubica para el año referido en 57 g/persona/día, estos valores se aproximan a los que prevalecían en el país hace 40 años.

Coincidimos con el Banco Mundial (5), cuando afirma que ingestas calóricas diarias de 2200 calorías por persona sólo son reales para poblaciones con actividad física muy ligera (y peso y talla bajos, ocasionados a la vez por bajas ingestas alimenticias); lo mismo puede afirmarse de requerimientos de proteína de 40 g/persona/día. En estas condiciones los pueblos no tienen futuro para avanzar hacia el progreso.

Proponemos, para nuestros pueblos, ingestas calóricas diarias, promedio, de 3000 calorías/persona y proteicas de 70 g, muy similares a las que tenían los países desarrollados hace 40 años. Sólo estos niveles garantizarían, de satisfacerse, que toda la población ingiera niveles adecuados para la actividad que requiere un pueblo que avance hacia el desarrollo y para garantizar el éxito de idóneos programas educativos y de salud. Sólo si se incrementa la disponibilidad de leguminosas de grano, la única fuente concentrada de proteínas a la que pudieran tener acceso las mayorías nacionales hoy sumidas en la miseria, a niveles de 21.9 kg/persona/año (60 g/persona/día) se podrían lograr metas como las propuestas, en un futuro cercano.

Como ya se indicó, el uso intensivo y masivo de las leguminosas, constituye un factor inestimable para el éxito de una estrategia de Desarrollo Agrícola en concordancia con las necesidades nacionales, y este último a su vez es indispensable para garantizar la satisfacción de los requerimientos de toda la población en lo que se refiere a alimentación, vestido, calzado, muebles, vivienda y artículos de papel y cartón. Además es indispensable también para generar fuentes de trabajo, tanto en el medio rural como en el urbano, única vía real para combatir la pobreza. Este planteamiento debería constituir un **Objetivo de Estado** de primer orden, de primerísima prioridad. Se cuenta con elementos suficientes para su viabilización y la existencia de un grupo interdisciplinario de investigación como lo es el Grupo de Investigación Interdisciplinaria en Canavalia (GIC), que cada vez amplía más su campo de estudio e investigación, constituye un esfuerzo y un aporte invalorable. Este grupo, con adecuado estímulo y apoyo puede y debe pasar a constituirse en el Grupo Interdisciplinario para el Estudio e Investigación en Leguminosas (GIESIL).

Dentro de la aberrante y absurda dependencia alimentaria que Venezuela ha padecido y padece, se han venido importando anualmente leguminosas de grano, leguminosas oleaginosas y harinas de leguminosas oleaginosas por un monto que ha oscilado entre 200 y 300 millones de dólares; en 1992 se importaron 558600 t de tortas y harinas oleaginosas por 129.4 millones de dólares, 115180 t de leguminosas de grano por 21.4 millones de dólares; 105000 t de soya por 47.2 millones de dólares y 2117 t de maní por 2.0 millones de dólares. Se propone al Gobierno Nacional destinar anualmente, por 10 años 60 millones de dólares al estudio, investigación y producción de leguminosas, destinando 15 millones de dólares a un grupo de investigación y estudio como el referido GIESIL y 45 millones de dólares al programa de producción. Concretando acciones como estas, Venezuela será pronto una Patria con futuro. ¿Lo entenderán así el Presidente del CONICIT, los Ministros de Agricultura y Cría, Sanidad y Asistencia Social y el propio Presidente de la República?. Ojalá que si.

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Effects of processing on antinutritional factors in legumes: The soybean case

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SUMMARY. The autor recounts his personal trail of research which has ultimately led to better understanding of the factors which contribute to the poor nutritive value of unheated soybeans. Among the 'techniques that were employed were the isolation of a lectin from raw soybeans, the use of affinity chromatography to remove the trypsin inhibitors, and the nutritional evaluation of soybean varieties which lacked the lectin or the Kunitz trypsin inhibitor. Based on a consideration of the results obtained by these experiments, it was estrimated that the trypsin inhibitors accounted for approximately 40% of the growth inihibiton on raw soy, of which two-thirds could be attributed to the Kunitz inhibitor and one-third to the Bowman-Birk inhibitor. The soybean agglutinin was deemed responsible for 50% of the inhibiton of growth, and the remaining 10% is most likely due to the poor digestibility of the undenatured protein.

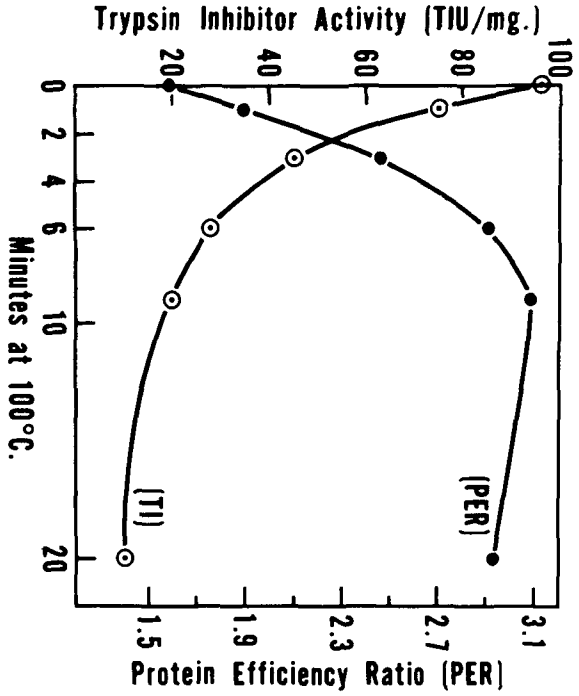
RESUMEN. Efecto del procesamiento sobre los factores antinutricionales de las semillas de leguminosa: el caso de la soya. El autor relata sus experiencias de investigación, que le han llevado finalmente a una mejor comprensión de los factores que contribuyen al pobre valor nutritivo de las semillas crudas de soya. Entre las técnicas empleadas figuran el asilameinto de la lectina de las semillas, el uso de cromatolografía de afinidad para remover los inhibidores de tripsina y la evaluación nutricional de variedades de soya desprovistas de lectina o del inhibidor de tripsina de Kunitz. En base al análisis de los resultados de dichos experimentos, se estimó que los inhibidores de tripsina contribuyen, aproximadamente, en un 40% a la inhibición del crecimento causado por la soya cruda; de este 40%, dos tercios pueden ser atribuidos al inhibidor de Kunitz y un tercio al de Bowman-Birk. La aglutinina de soya fue considerada como responsable del 50% de la inhibición del crecimiento. El restante 10% probablemente es debido a la pobre digestibilidad de las proteínas nativas.

BACKGROUND

My love affair with soybeans began more than four decades ago. Following my discharge from military service in 1946, I entered the PhD program in Biochemistry and Nutrition at the University of Southern California. After one year of intensive course work to get back on an academic track, I was made an offer I could not refuse. If I would agree to return to active duty, I would be assigned to the Quatermaster Corp in Chicago where, in addition to my military duties, I would be free to conduct my research for the PhD degree. One of the projects the Army was interested in at the time was the increased utilization of soybeans as a replacement for meat protein in army rations. One of the problems with the use of soybeans was the fact that, unless the soybean had been subjected to some from of processing, its nutritional value was very poor. I was asked to address this problem and to elucidate the basis for this phenomenon. Little did I realize at time that I was about to undertake a project which would become an almost life-time commitment.

Based on the early observation by Osborne and Mendel (1) it was already well known that the nutritive value of soybean protein poorly supported the growth of rats unless it had received some form of heat treatment. Also known at this time that soybeans in its raw state was a rich source of trypsin inhibitors (2). An example of the inverse relationship between the improvement in the nutritive value of soybean protein and the destruction of the trypsin inhibitor is shown in Fig. 1. This coupled with the observation that heat produced an improvement in the digestibility of the protein lead to what appeared to be the logical conclusion that the beneficial effect of heat treatment could be attributed to the destruction of these inhibitors that could otherwise interfere with the digestion of protein in the intestinal tract. Moreover, protein fractions which were highly enriched with trypsin inhibitor activity were capable of inhibiting the growth of rats, chicks, and mice (4-6).

FIGURE 1
Effect of heat treatment on the trypsin inhibitor activity and nutritive value of the protein as measured by the protein efficiency ratio (PER) rat assay. Taken from Rackis (3).



Despite these observatons it remained unclear why preparations of the trypsin inhibitor were capable of inhibiting growth even when incorporated into diets containing predigested protein or free amino acids (7). Such experiments obviously rule out an inhibiton of intestinal proteolysis as the sole factor responsabile for growth inihibition. Perhaps the most significant observaton that has ultimately led to better understanding of the mode of action of the soybean trypsin inhibitors was the finding that feeding of raw soybeans, or the purified inhibitors derived therefrom, caused an enlargement of the pancreas (8), which could be described histologically as hypertrophy as well as hyperplasia (9). Comcomitant with this increase in the size of the pancreas was an increase in the secretion of digestive enzymes, including trypsin, chymotrypsin, and elastase, and their ultimate excretion in the feces (10). Thus arose the hypothesis that the growth depression caused by the trypsin inhibitors was a consequence of an endogenous loss of amino acids in the form of enzymes being secreted by a hyperactive pancreas. Because pancreatic enzymes such as trypsin and chymotrypsin are particulary rich in the sulfur-containing amino acids, the effect of a hyperactive pancreas is to divert these amino acids from the synthesis of body tissue protein to the synthesis of these enzymes which are subsequently lost in the feces. The net effect is a loss in weight because of an exacerbation of an already critical situation with respect to soybean protein, which is inherently deficient in the sulfur-containing amino acids.

But several observations cast doubt as to whether the destruction of the trypsin inhibitor by heat was in fact the whole answer to the problem. The addition of a concentrate of the trypsin inhibitor to diets containgn heated soybeans at a level equivalent to the trypsin inhibitor activity of raw soy did not reduce the PER to the same level as the raw soy (7). In other words there was a definite indication that heat treatment was doing something more than just destroying the trypsin inhibitor. Furhermore, in a study involving 26 varieties of soybeans, there was no correlation between the protein efficiency ratio (PER) as measured in rats and their trypsin inhibitor content (11). Interestingly enough, there was, however, a significant correlation between the PER and the size of the pancreas. This would indicate that some factor other than the trypsin inhibitors must also be playing a role stimulating the growth of the pancreas.

In order to delineate more precisely the role of the trypsin inhibitor, instead of adding the trypsin inhibitor to heated soybeans, we decided to remove only the trypsin inhibitor from unheated soybeans to see what effect this would have on the nutritive value of the protein. This approach would then exclude any effect heat treatment might have other than its effect on the trypsin inhibitor *per se*. When rats were fed a raw soybean extract from which trypsin inhibitor activity had been removed by affinity chromatography on Sepharose-trypsin, it was found that only apaproximately 40% of the difference in PER's and pancreas weights between raw and heated soybean protein could be atributed to the trypsin inhibitor (12).

We were thus faced with the question as to what might be responsible for the approximately 60% of the growth inhibition produced by the ingestion of raw soybeans by the rat. It was this consideration which prompted our search for the possible presence of a growth inhibitor (s) which might account for the failure to explain the beneficial effect of heat treatment as being due solely to the trypsin inhibitors. As a first approach to this problem we simply wanted to see if one could show the presence of a toxic factor by the direct intraperitoneal injection of a crude extract of raw soybeans into rats. The injection of the pure crystalline preparation of the Kunitz trypsin inhibitor proved innocuous, whereas the crude extract with considerably less antitryptic activity was in fact quite toxic (13). These results made it quite clear that there was something other than trypsin inhibitor activity that was producing a toxic response in rats. Thus began our search for some yet unidentified toxic factor in soybeans.

The most promising candidate appeared to be agglutinins known to be present in legumes. As early as 1888 Stillmark had already shown that ricin, the toxic principle of the castor bean, displayed hemagglutinating activity (14). Later, in 1908, Landsteiner and Raubitschek (15) had shown that crude extracts of many edible legumes, including soybean, also had hemagglutinating activity. Little attention, however, was paid to the possibiity that these agglutinins, which would later be referred to as «lectins», were responsible for the poor nutritive

value of some of these legumes in their raw form. It wasn't until latter part of '49 and the early '50's that Jaffé directed our attention to the possibility that the toxicity of raw beans (*Phaseoulus vulgaris*) might be due to the presence of these hemagglutinins (16-18). These reports prompted us to attempt the isolation of what we thought could very well be the toxic factor in soybeans that we were looking for. Using fractional precipitation of the protein with ammonium sulfate, we found that the intraperitoneal toxicity was closely associated with hemagglutinating activity but was completely unrelated to trypsin inhibitor activity (19). We managed to purify this toxic factor with its associated hemagglutinating activity to the point of homogeneity as evidenced by moving boundary electrophoresis and sedimentation in the ultracentrifuge. We subsequently deterimned some of its more important physicochemical parameters including its molecular weight (20), amino acid composition and end group analysis (21), and the effect of chemical modificatoin (22). These studies revealed that the soybean agglutinin was comprised of several polypeptide chains and was a glycoprotein, chemical features which subsequently proved to be characteristic of most other lectins.

As an aside it should be menitoned that at this time I took the liberty of naming this protein «soyin», a term which was intended to denote its relationship to the other toxic hemagglutinins that were known at the time, such as «rincin» derived from the castor bean (*Ricinus communis*) and «abrin» from the jequirity bean (*Abrus precatorius*). It was subsequently brought to my attention that the name «soyin» had been prevoiusly used to denote the proteolytic activity in a crude extract of the soybean (23), although the enzyme responsible for this activity was, to my knowledge, never isolated or characterized. Nevertheless, in deference to these investigators, and in order to avoid confusion in the literature, we no longer used the term «soyin» in subsequent papers from our laboratory. This protein has since been simply referred to as the soybean agglutinin or SBA. It may be of intrest to note that at the time that we reported the isolation of the soybean agglutinin in 1952 (19), the term «lectin» had not been introduced into the literature until two years later by Boyd and Shapleigh (24).

Up to this point we had succeeded in showing there was a hemagglutinin in soybeans which was toxic when injected. It remained to be proven, however, that this protein was in fact responsible, at least to some extent, for the poor nutritive value of raw soybeans when consumed in the diet. To prove this point it became necessary to develop a method for the large-scale preparation of SBA which would enable us to incorporate it into a diet fed to rats. A labor intensive procedure involving salt and alcohol fractionation enabled us to obtain from each kg of raw soy flour at least 2 gm of a hemagglutin preparation which we judged to be about 78% pure, based on electrophoretic analysis, and virtually devoid of antitryptic activity. This preparation was added to a diet containing heated soy flour at a level which would provide the same level of hemagglutinating activity as an equivalent level of raw soy flour. The inclusion of SBA into a diet containg autoclaved soy flour accounted for about half of the growth depression obtained with the raw flour (25).

More recently we were afforded the unique opportunity of having made available to us by Dr. T. Hymowitz of the U. Ill. a soybean strain which lacked the gene for the soybean lectin. The results of feeding rats this particular strain of soybeans, which had less than 0.05% of the activity of a commercial variety, fully confirmed our previous experiment in which we added SBA to heated soybeans, that is, the lectin-free soybean showed an improvement in nutritive value that was about half that produced by heating alone (26). See Table 1. Note that there is very little difference in nutritive value between these two cultivars following heat treatment. It is important to point out that the trypsin inhibitor content of both soybean varieities was essentially the same, thus ruling out any effect due to the trypsin inhibitors.

TABLE 1
NUTRITIONAL VALUE OF «LECTIN-FREE» SOYBEAN CULTIVAR (T102) COMPARED WITH CONVENTIONAL COMMERCIAL VARIETY OF SOYBEANS (AMSOY) AS MEASURED IN RATS[a]

Soybean	Weight gain (g/21 days)	PER[b]	Lectin activity (HU/mg protein)[c]
Raw Amsoy	9.1	0.64	314×10^3
Raw T102	18.2	1.21	120
Heated Amsoy	68.9	2.58	0
Heated T102	73.4	2.73	0

a Data taken from Donatucci (26).
b Protein Efficiency Ratio.
c HU = hemagglutinating units.

In more recent years research by Pusztai and his group as well as other workers have further investigated the antinutritonal effects of the soybean lectin. Among their findings was the surprising fact that the soybean lectin was also responsible for pancreatic enlargement (27). In addition the soybean lectin induced a low level of circulating insulin, an increased rate of lipid metabolism, and cellular hyperplasia of the small intestine (28,29). The latter effect could also lead to an increase in an endogenous loss of protein resulting in an inhibition of growth. Other adverse effects attributed to the soybean lectin include an inhibition of the disaccharidases and proteases in the intestines (30) and an interference with absorption of non-heme iron (31).

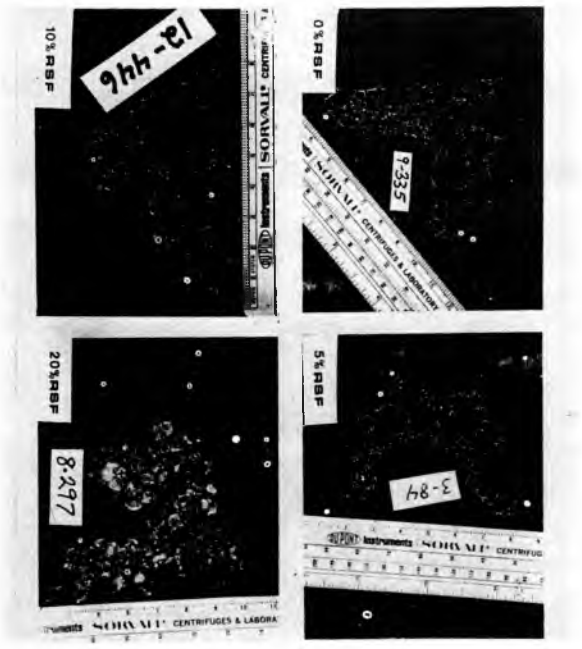
In addition to the trypsin inhibitors and lectin in soybeans another factor which must be taken into account is the digestibility of the protein itself. In order to dissociate the effect of the trypsin inhibitors on the digestibility of protein from the digestibility of the protein *per se, in vitro* digestibility studies were carried out on a crude extract of soybeans from which the trypsin inhibitors had been removed by affinity chromatography (12). A marked increase in the digestibility of

the soybean protein by trypsin was produced by heat treatment of the crude soybean extract. This increase, however, was much greater than the increase obtained with the unheated extract which was free of the inhibitor and may be attributed to the enhanced digestibility of the protein as a result of heat treatment. It should be mentioned that the resistance of native globulin proteins from the kidney bean to proteolytic attack had been reported prevoiusly from Jaffe's laboratory (32). It has been reported by Green *et al.* (33) that the presence of undigested protein in the small intestine can also cause an increase in pancreatic enzyme secretion. This it does in essentially the same way as the trypsin inhibitor, that is, by forming a stable enzyme-substrate complex with trypsin which results in removing the feedback inhibition of pancreatic secretion by trypsin.

Related to the increased proliferation of the pancreatic tissue evoked by the trypsin inhibitors is the finding that the long-term feeding of raw soyflour resulted in the production of adenomatous nodules on the pancreas (34). Prompted by these reports the USDA sponsered an in depth study in which rats were fed diets containinig various levels of raw soyflour so as to provide various levels of trypsin inihibitor activity (35). Fig. 2 compares the appearance of the pancreas of rats fed diets containing increasing levels of raw soy flour for a period of 18 months. A highly significant correlation was found between the incidence of nodules and the level of trypsin inhibitor activity in the diet.

FIGURE 2

Photograph of pancreas of rats fed diets containing increasing levels of raw soyflour (RSF) for a period of 18 months. Protein in diet was maintained at a constant level of 10% by appropriate mixtures of raw and heated soyflour. Taken from a study by Liener *et al* (35)



The effects of processing

Heat treatment. It is the relative ease with which the protease inhibitors and lectins are inactivated by moist heat treatment that has permitted the wide spread use of soybeans in animal and human diets. The general conclusion that can be drawn is that the extent to which the trypsin inhibitors and lectins are destroyed by heat treatment is a function of temperature, duration of heating, particle size, and moisture conditions. All of these factors are carefully monitored and controlled during the commercial production of soybean products in order to insure a product having maximum nutritional value. It is important to point out, however, that excessive heat treatment should be avoided in order to prevent damage to the nutritonal value of the protein. In seeking a compromise between these two apposing effects, it is not surprising that one generally finds small but measurable amounts of trypsin inhibitor activity in a variety of products containinig soy protein as the main source of protein (36).

Although these relatively low levels of trypsin inhibitor activity probably pose little risk to the general population, there are certain segments of the population that might be more vulnerable to the adverse effects of even low levels of the trypsin inhibitor. Since residual trypsin inhibitor activity may still remain in soy based infant formulas (37), infants who are fed soy milk for prolonged periods because of an allergy to cow's milk could be at risk. Another population group that might be at risk by continuous dietary exposure to the trypsin inhibitors are those individuals who are vegetarians, either by choice or culture, and most often choose legumes such as soybeans as a meat replacement. A similar situation would prevail in those individuals suffering from hyperlipidemia or hypercholesterolemia who have been advised to replace the animal protien with soybean protein in order to reduce the level of blood cholesterol.

Because of the compact structure of the Bowman-Birk inhibitor (BBI) and its stability towards heat in its purified state (38), it has been generally assumed that most of the residual trypsin inihibitor activity found in heat processed soybean products is due to this inhibitor. However, using a technique which serve to differentiate between the Kunitz inhibitor and BBI, it was the latter that was more readily destroyed than the Kunitz inhibitor (39). This difference in the heat resistance between the Kunitz inhibitor and BBI assumes added significance in view of reports of the anticarcinogenic properties of BBI (40). Thus, if one is prompted to preserve the BBI content of soybean products as a means of preventing cancer, more careful attention will have to be paid to the processing conditions used in producing such products.

The inactivation of the soybean lectin by moist heat treatment closely parallels the destruction of the trypsin inhibitors in soybeans. Since the soybean lectin is quite resistant to inactivation by dry heat treatment (41), this may explain why low but measurable levels of lectin activity were detected in a number of

soy containing products (42). It is doubtful, however, whether the final concentration of lectin in these products are such that they would pose a risk to human health.

Germination: Although the germination of soybeans has been reported to result in an improvement in the nuritive value of the protein, this effect appears to be unrelated to the level of trypsin inhibitor in the germinated bean (43). As far as the soybean lectin is concerned, germination is accompanied by a rapid disappearance of hemagglutinating activity (44). This perhaps may be one of the factors what accounts for the improved nutritional value of the germinated bean.

Traditional soybean dishes: Since the preparation of tofu, soymilk, and fermented dishes such as tempeh and natto generally involves the cooking or streaming of soybeans during or piror to extraction with water or fermentation, such dishes are generally quite low in trypsin inhibitor activity (45).

Chemical Treatment: Although heat is an effective and simple means of inactivating the trypsin inhibitors of soybeans, as already indicated, it carries with it the risk that excessive heat may damage the protein. Adjunct treatment with various thiol-containign chemicals, such as N-acetyl-cysteine and glutathione has been found to facilitate inactivation at lower temeratures (46). This inactivation is most likely a consequence of the interaction of the disulfide bonds of the trypsin inhibitors through the formation of mixed disulfides. The treatment of soy products with sodium sulfite, a reagent which is known to cleave disulfide bonds, likewise served to reduce the temperature necessary to inactivate the trypsin inhibitors (47).

Genetic Variants: Numerous studies have beeen devoted to a search for varieties of soybeans that might be low in trypsin inhibitor content, but, as already noted (11), there appears to be little correlation between trypsin inhibitor content and the nutritonal value of the protien. Hymowitz and coworkers have succeeded in identifying several isolines which lacked the Kunitz trypsin inhibitor but retained about 50% of the trypsin inhibitor activity of a common commercial variety of soybeans (48). This remaining activity was found to be entirely due to BBI. Feeding studies with several species of animals showed that the soybean isoline with reduced trypsin inhibitor activity supported better growth than raw soybeans containing the Kunitz inhibitor as well (48,49). An example of the performance of rats in terms of PER and the size of the pancreas when placed on a diet containing the soybean lacking the Kunitz inhibitor is shown in Table 2. From these data it may be calculated that approximately one-third of the growth inihibition and pancreatic enlargement produced by raw soy still remains after removal of the Kunitz inhibitor and may be presumed to be due to BBI. Heat treatment, however, still proved to be the most effective means for enchancing the nutritional value of the protein. The practical implication from these studies is the fact that milder heat treatment is needed to achieve near zero level of TI activity with the isoline laking the Kunitz inhibitor than with standard varieties of soybeans (49,51).

TABLE 2
BIOLOGICAL EVALUATION OF A SOYBEAN LINE (PI 147440) WHICH LACKS THE KUNITZ SOYBEAN INHIBITOR[a]

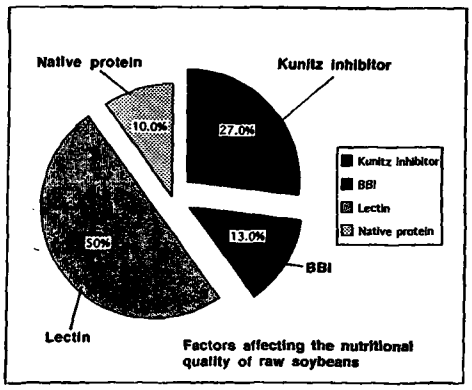
Soybean	TI activity[b]	PER[c]	Pancreas wgt.[d]
Unheated soy flour	100	0.98 (100)	0.61 (100)
Heated soyflour	5	2.39 (0)	0.40 (0)
Unheated PI 157440	40	1.44 (32)	0.54 (33)
Heated PI 157440	5	2.42 (102)	0.42 (90)

a Data taken from Tarcza (50).
b As percent of unheated soy flour.
c Protein efficiency ratio. Values in parentheses denote % of the growth inhibition obtained with unheated soy flour taken as 100%.
d Expressed as % of body weight. Values in parentheses denote % of the increase in weight of pancreas from unheated soy flour taken as 100%.

CONCLUSION

After decades of research the available evidence now permits an approximation as to how much of the beneficial effect of processing of soybeans is due to the inactivation of each of the antinutritional components shown in Fig. 3. The contribution of the trypsin inhibitors to the overall effect is about 40% of which two-thirds is due to the Kunitz inhibitor and one-third to BBI. The soybean lectin accounts for about 50% of the effect, and the remaining 10% must be due to the enhanced digestibility of denatured protein.

FIGURE 3
Pie chart showing the estimated contribution of the various antinutritional factors to the overall effect produced by raw soy beans. The inactivation of these factors by processing is responsible for the beneficial effect of heat treatment.



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