CHAPTER 3

Hemagglutinins

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I. INTRODUCTION

The extracts of many plants have the property to agglutinate red blood cells caused by some remarkable proteins called "phytohemagglutinins" or "lectins."* They are found mostly in seeds from which they may be extracted by water or salt solution; they may also exist in leaves, bark, roots, tubers, latex, etc. Agglutinin-containing plants have been found in many botanical groups including mono- and dicotyledons, molds and lichens, but most frequently they have been detected in *Leguminosae* and *Euphorbiaceae* (Tobiska, 1964).

The first description of a phytohemagglutinin was given by Stillmark (1889), who studied the toxicity of castor beans and press cake from the

*The terms phytohemagglutinins, phytagglutinins, and lectins are used interchangeably.

production of castor oil. From his very thorough investigation he concluded that the toxic action was due to a protein which he called "ricin" and which he showed to be capable of agglutinating the red cells from human and animal blood.

Several other toxic plant proteins have been described in the following years after the discovery of ricin. Many have never been studied in much detail. The relative facility with which the castor beans can be obtained and the strong toxicity of ricin were probably the reason why more investigators were attracted to this rather than to similar but less easily available plants.

The early literature on the toxicological properties of the plant hemagglutinins has been reviewed by Ford (1913) and Brocq-Rousseu and Fabre (1947). More recent reviews emphasizing the physicochemical properties and nutritional significance of the phytagglutinins may be found in papers by Liener (1962, 1964).

The first antitoxins were prepared using ricin and abrin, the toxin from the seeds of *Abrus precatorius* by Ehrlich (1891a,b). He not only demonstrated the neutralizing action of the serum of mice immunized against these toxins when mixed with a solution of the corresponding seed extract, but also the specificity of this reaction because antiricin serum would not act on abrin and vice versa.

Landsteiner and Raubitschek (1908) observed that extracts from many edible crude legume seeds would likewise agglutinate red blood cells, but no toxic action was detected in these seeds at that time. At the same time they established that the relative hemagglutinating activities of various seeds were quite different when tested with blood cells from different animals and compared this specificity with that of the antibodies of animal blood serum. The name agglutinin was first proposed by Elfstrand (1897) for the phytohemagglutinins and only later was its use extended to immunoagglutinins.

The role which the plant toxins played in the early phase of immunological investigations is exemplified by a quotation from Ford (1913):

> The two past decades have seen investigations of the plant poisons along many lines, both chemical and immunological, chiefly in the hope of elucidating some of the important and difficult problems of the bacterial toxins and their antitoxins. The ease with which the plant poisons can be obtained in quantity, their stability, the readiness with which they lend themselves to test tube experiments, and the definite reactions which they produce in animals render this group particularly valuable for immunological experiments. Already some of the important problems in this field have been solved or their solution hastened by these investigations. Interest in the study of this group of poisons is constantly increasing, however, and the next few years are likely to see more complete and elaborate investigations and results of the most far-reaching importance.

Several plant agglutinins, ricin, abrin, and phasin from navy beans were commercially available at that time.

Renkonen (1948) and Boyd and Reguera (1949) independently reported on the existence of plant agglutinins which exhibit specificity toward samples of human blood belonging to different blood groups. This discovery marked a new era in the investigations of seed agglutinins in which the immunological outranked the toxicological aspects.

The application of the erythroagglutinating action for the separation of red blood cells from leukocytes led Nowell (1960) to the important observation of the mitosis-inducing activity of the kidney bean agglutinin. A new field of investigation is based on this discovery which led to the development of simple methods for the study of human chromosomes and its clinical application. The literature on the mitogenic action of the phytohemagglutinins have been reviewed by Robbins (1964). Mitogenic activity has been detected in several different plant extracts (Krüpe *et al.*, 1968).

II. SPECIFIC AND NONSPECIFIC HEMAGGLUTININS

All known phytohemagglutinins show differences with respect to their activity on blood of different animals as has been observed by Stillmark (1889) for ricin and by Elfstrand (1897) for crotin from *Croton tiglium*. The data of Table I demonstrate the specificity of extracts from the seeds of different legumes for the blood of certain animal species.

TABLE I

AGGLUTINATION OF BLOOD FROM DIFFERENT ANIMALS BY LEGUME EXTRACTS[a]

	Seed extracts			
Blood	Beans	Peas	Lentils	Sweet peas
Human	800	40	30	20
Horse	16,000	128	64	128
Rabbit	8,000	1,000	2,000	200
Sheep	1,600	4	–	–
Pigeon	32,000	–	–	400
Carp	800	400	200	10
Frog	400	80	–	8

[a] Landsteiner and Raubitschek, 1908. The highest dilutions of the respective extracts still active in the agglutination test are reported.

The observation of Renkonen (1948) and of Boyd and Reguera (1949) on the different activities of some plant agglutinins toward the blood of humans belonging to different blood groups was the reason why the distinction between "specific" and "nonspecific" agglutinins was made. These terms have produced some confusion because they obscure the fact that the nonspecific agglutinins, although acting on the cells of any human blood group, nevertheless show very characteristic differences when brought into contact with erythrocytes from different animal species. Only a fraction of the known agglutinins exhibit blood group specificity. They have special interest for the hematologist and several excellent reviews have been published on this aspect of the subject (Krüpe, 1956; Bird, 1959; Saint-Paul, 1961; Boyd, 1963; Tobiska, 1964). Blood group specific and nonspecific agglutinins may exist together in some plants like navy peas (*Phaseolus vulgaris*) (Toms and Turner, 1965) and *Vicia cracca* (Asberg *et al.* 1968).

Boyd and Shapleigh (1954a) proposed the name "lectins" which is derived from the Latin word *legere*, to choose, in order to point to the specificity of the phytoagglutinins. This term has sometimes been used exclusively for the blood group specific agglutinins although Boyd and Shapleigh apparently did not intend to imply this distinction.

It is important to realize that the agglutinins differ widely from each other, that they exhibit considerable specificity, and that different agglutinins should be regarded as distinct molecular species with different chemical and biological characteristics.

In some cases the agglutinating action is inhibited by specific sugars as Morgan and Watkins (1953) had first observed. The receptor groups of the erythrocytes possess carbohydrate moieties. The inhibition of the agglutination is explained by the interaction of the sugars with the reactive site of the lectins in such a manner that they can no longer react with their specific receptor groups (Krüpe, 1956).

Of practical interest is the use of some lectins for anthropological and clinical detection of blood groups in humans (Boyd, 1963), for the detection of subjects who excrete blood group specific substance in the saliva, the so-called "excretors" (Boyd and Shapleigh, 1954b), and for the differentiation of animal and human blood (Reimann and Popwasiloff, 1960).

Toxicity has not been reported for any of the blood group specific lectins with the exception of the soybean agglutinin which shows specificity under special experimental conditions (Bird, 1953). The number of plants known to exhibit hemagglutinating activity has grown rapidly since the discovery of blood group specificity in lectins. Tobiska (1964) lists over 500 species of agglutinin-containing plants. Most of these have never been tested for toxicity.

The occurrence of agglutinins in plants is probably controlled by genetic factors (Schertz *et al.*, 1960). Agglutinin-free kidney bean varieties have been observed (Tobiska, 1964). The environment may modify the strength of agglutinating activity in plants as has been shown by Tobiska and Lhotecka-Brazdova (1960) who studied the action of fertilizers on the hemagglutinating properties of kidney beans. Lalaurie *et al.* (1965) observed that plants of *Ulex parviflorus* exposed to sunlight had less hemagglutinins than those kept in the shadow. Inhibitors occurring in the plant together with the lectins may prevent full activity (Renkonen, 1960).

III. COMPARATIVE TOXICITY OF AGGLUTININS

Since the first description of a plant toxin with hemagglutinating activity, ten toxic phytohemagglutinins have been described. From the date of Table II it can be seen that plants belonging to two families, i.e., *Euphorbiaceae* and *Leguminosae,* are known to contain toxic agglutinins. It is difficult to compare from the published reports the degree of toxicity of the various hemagglutinin preparations. Frequently the degree of purity of the fractions was unknown, and different species or strains of animals and different techniques of application were used in the toxicological studies. No systematic investigation on the comparative toxicity of the hemagglutinins have been reported nor has the relationship be-

TABLE II

TOXIC PHYTAGGLUTININS

Name	Plant	Family	Reference
Ricin	*Ricinus communis*	Euphorbiaceae	Stillmark, 1889
Crotin	*Croton triglium*	Euphorbiaceae	Elfstrand, 1897
Curcin[a]	*Jatropha curcas*	Euphorbiaceae	Siegel, 1893
Crepitin	*Hura crepitans*	Euphorbiaceae	Richet, 1909
Robin	*Robinia pseudacacia*	Leguminoseae	Power and Cambier, 1890
Abrin	*Abrus precatorius*	Leguminoseae	Warden and Waddell, 1884
Concanavalin A	*Canavalia ensiformis*	Leguminoseae	Sumner and Howell, 1936
Soybean agglutinin	*Glycine max*[b]	Leguminoseae	Liener and Pallansch, 1952
Phaseolotoxin A	*Phaseolus vulgaris*[b]	Leguminoseae	Jaffé and Gaede, 1959
Field bean hemagglutinin	*Dolichos lablab*[b]	Leguminoseae	Salgarkar and Sohonie, 1965a

[a]Hemagglutinating activity of curcin has not been described in the literature, but has been observed in the author's laboratory with guinea pig, rat, and chick blood.

tween the susceptibility of the blood cells of different animals to agglutination by certain lectins and the toxicity been investigated.

Orally ingested ricin is more toxic for horses and rabbits than for chicks (Bierbaum, 1906). Field (1910) reported lethal doses of a highly active ricin preparation injected intramuscularly which ranged from 0.0001 mg/kg for rabbits to 0.03 mg/kg for the goat. Toxicity of ricin for mice is different according to strain, age, nutritional conditions, and individual susceptibility (Ehrlich, 1891a). Elfstrand (1897) stated that ducks and chicks are more resistant to crotin than all other animals tested. Honavar *et al.* (1962) observed that kidney bean agglutinin inhibits the growth and causes death of rats when fed at a 0.5% level in the diet. In a similar experiment with chicks, Wagh *et al.* (1963) noted that growth decrease was much less than in rats and no lethal action was detected. The susceptibility of mice from different strains to injected black bean agglutinin was different (Jaffé 1962). The influence of the age is apparent from the observations of Richet (1910) who observed that young dogs were more resistant to the toxic action of crepitin than older animals.

From the doses of ricin effective in killing about half of the injected mice reported by Schöne (1958) and Ishiguro *et al.* (1964a) and those observed for the phytohemagglutinin from black beans by Jaffé (1960), it seems that ricin is at least 1000 times more toxic than the bean agglutinin. The soybean lectin injected by Liener and Pallansch (1952) into rats and the black bean agglutinin used by Jaffé (1960) in mice produced death at very similar levels. Honavar *et al.* (1962) fed the lectins from kidney beans and from black beans to rats and found that the former was more toxic than the latter. A phytoagglutinin isolated from white peas by Huprikar and Sohonie (1965) failed to exhibit any toxic action when fed to rats at a level of 1% at which soybean and kidney bean agglutinins are definitely toxic. Evidently, there exist very large differences in toxicity between the various seed agglutinins, and some of them may well be completely devoid of any poisonous action.

IV. PREPARATION AND PROPERTIES

Only six of the ten agglutinins listed in Table II have been purified to a reasonable degree. Their chemical and biological properties are still only partly known.

A. Ricin

Ricin had been purified by Osborne and co-workers (1905), Karrer *et al.* (1924), and others before the introduction of modern methods of

protein chemistry, and they had established its protein nature. It was later crystallized by Kunitz and McDonald (1948), who observed, however, that the material, even after several recrystallizations, consisted of more than one compound. Corwin (1961) stated that for the crystallization of ricin the presence of another substance, the ricinus allergen, is indispensable, since both compounds form a single saltlike complex. Kabat *et al.* (1947), Schöne (1958), and Ishiguro *et al.* (1964a) have also shown that crystalline ricin is not hemogeneous and that it can be fractionated further. The last group of investigators separated from crystallized ricin a fraction called ricin D, which was obtained in crystalline form and was considered to be pure (Ishiguro *et al.*, 1964b).

Kunitz and McDonald (1948) precipitated the active compound from the water extract of defatted castor beans by saturation with sodium sulfate. Moulé (1951a) used fractional precipitation with ammonium sulfate and found that the hemagglutinating and toxic activity was concentrated in the fraction precipitating between 33 and 50% saturation. Crystallization could be achieved from the crude extract, or from a solution of the precipitate formed by saturation with sodium sulfate, by storage for several weeks at about 5°C in the presence of this salt (Kunitz and McDonald, 1948).

For further purification, electrophoretic and chromatographic techniques have been used. Schöne (1958) has applied preparative paper electrophoresis for the purification of his fractions. Dirheimer and Haas (1965) used fractionation by ammonium sulfate precipitation and chromatography on Sephadex G-100 and obtained a preparation which was homogeneous by hydroxyl-apatite chromatography, electrophoresis on cellulose acetate, and by ultracentrifugal analysis. Janssen (1964) applied chromatography on CM-cellulose and on Sephadex G-75 for purification. Waller *et al.* (1966) obtained two fractions with hemagglutinating and toxic properties by heating a ricin solution to 65°C at pH 5.0 for the elimination of inert proteins and subsequent chromatography on Amberlite CG-50.

Amino acid analysis of ricin has been reported by Karrer *et al* (1924), Moulé (1951b), Holasek *et al.* (1955), and Schöne (1958), but the results are contradictory. Isoleucine and methionine were detected by Holasek *et al.* (1955) as N-terminal amino acids by the use of the dinitrofluorobenzene method. Schöne, however, could not detect any endgroups with this same method. The extinction coefficient at 280 mμ is higher for ricin than for any other castor bean protein (Janssen, 1964).

Ishiguro *et al.* (1964a) described the separation of a highly toxic, but nonagglutinating fraction, ricin D, from castor bean proteins by chromatography on hydroxyl-apatite and DEAE-cellulose. A nontoxic agglutinating compound was also obtained and called castor bean hemagglutinin

(Takahashi *et al.*, 1962a). The physical properties of both compounds have been reported and are included in Table III. No detailed investigations on the biological and chemical properties have yet been published so that a comparison with the results of other authors is difficult. These observations are important because the identity of the toxic and hemagglutinating compounds of ricin has not been proved, and several claims have been made that one action can be abolished without the other. Thus, treatment of toxic ricinus seed extracts with ninhydrin has been used to eliminate the toxicity but not the agglutinating activity (Corwin, 1961). Differences in heat inactivation of the toxic and hemagglutinating actions were reported by Olmer and Sauvan (1909). According to Fuchs and Falkensammer (1939), digestion with pepsin destroys more rapidly the hemagglutinating than the toxic activity, but Osborne *et al.* (1905) found both activities were slowly and simultaneously destroyed by trypsin. Clarke (1954) titrated ricin solutions with rabbit and goat antiricin serum, and found that the toxicity was reduced to about 1/1000, while the agglutinating action on red cells was even enhanced in some experiments. Kabat *et al.* (1947), on the other hand, observed that both activities were reduced simultaneously by the antisera from rabbits, goats, or horses. Ehrlich (1891a), in his classic work on the preparation of ricin antitoxin, used successfully the supression of the agglutinating action of the antisera as a measure of antitoxic activity.

The process of hemagglutination is still little understood, a fact which makes the interpretation of the contradictory results difficult. Kabat *et al.* (1947), for example, have obtained fractions by precipitation with acetone which had as little as one-fortieth of the hemagglutinating power of purified ricin. Addition of serum or of 0.5% formol solution restored hemagglutinating potency. They concluded that hemagglutinating activity may be a property of ricin itself but may be influenced by the presence of other substances. Krüpe (1954) observed the disappearance of hemagglutinating action in an extract from seeds of *Sophora japonica* which was restored spontaneously after several days storage or could be demonstrated when the agglutinin test was performed in the presence of 20% serum albumin, 8% gelatin solution, or AB serum, resembling in this aspect the so-called incomplete antibodies such as the isoantibodies anti-Rh. The results of Mourgue *et al.* (1958) and of Waller *et al.* (1966) suggest the presence of two compounds with toxic and hemagglutinating properties in crude ricin which can be separated by chromatography. Toxic and nontoxic compounds may exist in crystalline ricin which are indistinguishable by immunological reactions and by electrophoretic and ultracentrifugal methods. Kabat *et al.* (1947) came to this conclusion through the comparison of the toxicity of certain ricin frac-

TABLE III

PHYSICAL CONSTANTS OF SOME PHYTOHEMAGGLUTININS

Plant	Name	Sedimentation constant ($S_{20,w}$)	Molecular weight	Isoelectric point	Reference
Castor bean (*Ricinus communis*)	Ricin	4.8	85.000	5.4	Kabat *et al.* (1947)
	Ricin	3.9	36.000	–	Kunitz and McDonald (1948)
	Ricin	4.78	70–75.000	–	Schöne (1958)
	Ricin		40.000		Janssen (1964)
	Ricin D	4.64	62.000	5.9	Ishiguro *et al.* (1964b)
	Castor bean hemagglutinin	6.39	98.000	–	Takahashi *et al.* (1962b)
Soybean (*Glycine max*)	Soybean hemagglutinin	6.4	105.000	6.1	Pallansch and Liener (1953)
			110.000		Lis *et al.* (1966)
Red kidney bean (*Phaseolus vulgaris*)	PHA	6.5	128.000	6.5	Rigas and Osgood (1955) Rigas and Johnson (1964)
Black bean (*Phaseolus vulgaris*)	Phaseolotoxin A	5.9	126–130.000	4.9	Jaffé and Gaede (1959) Camejo (1964)
Yellow wax bean (*Phaseolus vulgaris*)	Yellow wax bean hemaglutinin	5.37	121–132.000	5.5	Takahashi *et al.* (1967)
Jack bean (*Canavalia ensiformis*)	Concanavalin A		96.000	–	Sumner and Howell (1936)
	Concanavalin A	6.0	71.000	–	Olson and Liener (1967a)
Robinia pseudacacia		4.39	–	5–6	Bourrillon and Font (1968)

tions with the crystalline ricin. They could achieve a partial separation by means of fractional crystallization. From their quantitative immunochemical assays with a large number of rabbit antisera and several horse and goat antisera, they found strong evidence that hemagglutinating power and toxicity are properties of the same molecule.

The resistance of ricin to digestion had been observed by Stillmark (1889) and by many later investigators. Karrer *et al.* (1924) noted that after 5 months contact with pancreatic juice only about 90% of the proteins of a purified ricin preparation had been hydrolyzed. The remaining toxin could be isolated, and toxic power was the same as that of the starting material. Ricin has been detected in 21 different species and varieties of *Ricinus* by Agulhon (1914).

B. Kidney Bean Agglutinin

The feeding of raw kidney beans (*Phaseolus vulgaris*), as part of a diet for experimental animals, causes rapid loss of weight and death, but the heated seeds have no similar effect. Johns and Finks (1920a,b) and Berczeller (1922) explained this effect by the low digestibility of the raw beans. The existence of a bean agglutinin had been reported by Landsteiner and Raubitschek (1908), and Wienhaus (1909) studied it in some detail but failed to detect significant toxic action, probably because he supposed that it were similar to ricin in poisonous activity. He proposed the name of phasin for this agglutinin which since then has sometimes been used to designate nontoxic plant agglutinins. Lüning and Bartels (1926) were probably the first to relate the toxic action of beans with the agglutinin content. Jaffé (1949) again observed the toxic action of raw beans and showed that it could not be explained by poor digestibility or by the presence of trypsin inhibitors (see Chapter 2) because enzymatically digested casein when added to the toxic diet did not improve the performance of the experimental animals. No cyanogenic glycosides were found in toxic amounts in *Phaseolus vulgaris* (Jaffé, 1950; Montgomery 1964; see also Chapter 5).

A protein active in the agglutination of red blood cells and toxic when injected into mice was prepared by Jaffé and Gaede (1959) from black beans by ammonium sulfate fractionation and called phaseolotoxin A. This name was chosen to call attention to the toxic properties not recognized previously by most investigators in the so-called phasin. Rigas and Osgood (1955) did not observe poisonous properties in a bean agglutinin they had prepared. Jaffé (1960) found that a bean fraction obtained according to the procedure of these authors agglutinated red cells and was toxic when injected into mice in rather large amounts. Honavar *et al.* (1962) purified a hemagglutinin from kidney beans and

observed its marked toxicity in rats. It inhibited growth completely when fed at a 0.5% level and caused the death of the animals within 2 weeks. This activity was completely destroyed by boiling a solution of the agglutinin for 30 min. The wax bean agglutinin isolated by Takahashi *et al.* (1967) was homogeneous by ultracentrifugal analysis and electrophoresis on polyacrylamide gel. Its physicochemical properties differ somewhat from those described for other bean lectins (Table III).

The hemagglutinating action of bean lectins has been studied frequently. Goddard and Mendel (1929) prepared a bean fraction and observed that the agglutinating activity was inhibited by egg white. About 8 times more lectin was fixed to the blood cells than the minimum quantity causing agglutination. Coulet (1954), Saint-Paul *et al.* (1956), Tobiska and Widermann (1959), and others have likewise studied the hemagglutinating and immunological properties of partially purified kidney bean lectins without exploring their toxicity.

Several investigators have observed that more than one hemagglutinating fraction may be present in kidney bean extracts. Pierkarski (1957) prepared three, and Jaffé and Gaede (1959) isolated two fraction possessing hemagglutinating and toxic properties. Prager and Speer (1959) separated a crude bean extract into three agglutinating fractions by chromatography on DEAE-cellulose. Jaffé and Hannig (1965) demonstrated by immunoelectrophoresis that at least two different hemagglutinins exist in a black bean extract which were separated by ammonium sulfate precipitation and free flow electrophoresis, and Takahashi *et al.* (1967) separated two hemagglutinating proteins from wax beans by DEAE-cellulose chromatography. The simultaneous presence of lectins with different specificities, i.e., a nonspecific and an anti-A + B agglutinin, was observed in a variety of *Phaseolus vulgaris*, the navy peas (Toms and Turner, 1965).

Kakade and Evans (1965a,b, 1966) have announced the achievement of a partial separation of the toxic from the hemagglutinating activities of navy beans and concluded that both actions may be due to two different substances. The fractions obtained by these authors still exhibit both activities, one being relatively more toxic and the other more active in agglutination. Stead *et al.* (1966) separated several fractions from crude extracts of round yellow beans by chromatography on DEAE-cellulose. The fraction containing the bulk of the hemagglutinating activity was less toxic than a fraction possessing little hemagglutinating power. From these results they conclude that the agglutinin and the toxic compounds are not identical. Jaffé (1962) has prepared three fractions from black beans in which the toxic and hemagglutinating activities were not directly proportional and proposed that several more or less toxic lectins may exist in beans.

Rigas *et al.* (1966) demonstrated that a bean agglutinin which was homogeneous by eleven different criteria, including several types and conditions of electrophoresis, gel filtration, chromatography, and ultracentrifugation and which had only one N-terminal amino acid, alanine, dissociated slowly into eight subunits when kept in 8 *M* urea. These could be separated by starch gel electrophoresis. Chromatography of the lectin on the cation exchange resin IRC-50 resulted in the appearance of several fractions differing in their physical and biological properties, amino acid composition, and subunit ratio. It was suggested by the authors that dissociation into subunits and recombination occurs during the chromatography, that different subunits may be responsible for the hemagglutinating, mitogenic, and toxic activities, and that inactive subunits may also be present.

Nungester and Van Halsema (1953) found certain *P. vulgaris* extracts to interact with Flexner Jobling carcinoma cells of the rat, and Steck and Wallach (1965) made similar observations with Ehrlich ascitis carcinoma of mice. Liener and Seto (1955) demonstrated that repeated injections of soybean hemagglutinin into rats inoculated with Walker tumor caused a delay in the appearance of and reduction in the size of the tumors. The same effect was observed in the pair fed controls and was therefore related to the reduced food intake rather than to a specific tumor-inhibiting action.

The mitosis stimulating activity of kidney bean agglutinin preparations has already been mentioned (see Robbins, 1964). This action can be detected in highly purified bean lectin according to Rigas *et al.* (1966), who discuss the question whether the mitogenic and the hemagglutinating factors are identical.

Phaseolus multiflorus, the runner bean, has toxic and agglutinating properties (de Muelenaere, 1965) but the active principle has not been purified. The lima bean (*Phaseolus lunatus*) lectin has been studied by Boyd *et al.* (1955). It acts specifically on human group A blood cells. These seeds seem to have no toxic action in rats comparable to that of kidney beans (Jaffé, 1950; de Muelenaere, 1965).

C. Soybean Agglutinin

The enhancement of the nutritional value of soybeans produced by heating has been known since Osborne and Mendel's observations in 1917, but the reason for this effect of heat is not yet fully understood (see Chapter 2, VII). Evidence that part of the toxic action of raw soybeans is related to the lectin came from the work of Liener and his co-workers who isolated a hemagglutinating protein from raw soy flour and extensively studied its physical, chemical, and biological aspects.

In the earlier work this protein was called soyin but, in the later papers, the name soybean hemagglutinin was preferred since a proteolytic enzyme had been called soyin at an earlier date. The agglutinin was homogenous by chromatography on DEAE-cellulose, electrophoresis at various pH values, starch gel electrophoresis, and ultracentrifugation (Pallansch and Liener, 1953). From the investigation of the N-terminal amino acids it appeared to consist of two peptide chains. Glucosamine was found in this compound in rather large amounts (Wada *et al.*, 1958). In addition to the hemagglutinin described by Liener and his co-workers three other minor components have been separated from a crude soybean extract by Lis *et al.* (1966); all four have agglutinating properties and are similar in electrophoretic behavior and in chromatography on CM-cellulose or on calcium phosphate. Separation was attained by chromatography on DEAE-cellulose under strictly controlled conditions. The fractions are alike in amino acid composition and contain neutral sugars and glucosamine in different amounts.

The toxicity of the soybean agglutinin has been studied under various conditions by Liener and Pallansch (1952) and Liener (1951, 1953). The LD_{50} for young rats is about 50 mg/kg. Added to a diet containing autoclaved soybean meal at the level of 1%, it depresses growth to about 75% of that of the controls, and food intake is reduced. About half of the growth depression caused by raw soy meal could be attributed to the action of the lectin. Its destruction by heat is associated with an improvement of the nutritive value of soybeans (Liener and Hill, 1953). The amount of the agglutinin in soybean flour has been estimated by a immunochemical procedure and found to be about 3% (Liener and Rose, 1953).

Although the lethal doses of the soybean and kidney bean agglutinins administered by the intraperitoneal route are similar (Liener and Pallansch, 1952; Jaffé 1960), raw kidney beans are more toxic for growing rats than raw soybeans. The former cause rapid loss of weight and death but the latter permit fair growth of the experimental animals. The same difference can be observed when the isolated lectins are compared, that from beans being highly toxic and causing death when fed to young rats (Honavar *et al.* 1962), but the soybean lectin having no lethal action when administered to rats by stomach tube at a level of 500 mg/kg (Liener and Rose, 1953). Different susceptibility to digestion could account for this difference. This is suggested by observations on the action of pepsin which inactivates soybean agglutinin rapidly according to Liener (1958a), but the kidney bean agglutinin is rather resistant and is not completely inactivated after 6 days' digestion by this enzyme (Goddard and Mendel, 1929). Hemagglutinating activity could be detected in the feces of rats after they had been fed raw black beans or kidney beans but not after the ingestion of soybeans (Jaffé and Vega

Lette, 1968), which indicates that the bean agglutinin is less susceptible to the action of digestive enzymes *in vivo* than that from soybeans.

Stead *et al.* (1966) performed a chromatographic separation of soybean proteins on DEAE-cellulose. The hemagglutinating activity and toxicity were concentrated in one of the peaks in the eluate, but the material from another peak was also toxic when injected into rats although devoid of significant hemagglutinating action. Birk and Gertler (1961) observed that most of the hemagglutinating material can be extracted from raw soybean meal, but the nutritional quality of the residue is not much improved. Although the toxic property of the soybean agglutinin is well established, observations on the poor nutritional value of the uncooked soybeans point clearly to the existence of still other toxic factors in this material (see, for example, the protease inhibitors, Chapter 2,VII,A).

Soybean extracts agglutinate rabbit blood cells rapidly. Rat erythrocytes are agglutinated only by large amounts of the agglutinin, and blood cells from sheep and calves were completely refractory to agglutination (Liener and Pallansch, 1952). Bird (1953) stated that soybean extracts contain cold agglutinins active on human blood cells at low temperature.

D. Other Agglutinins

Crotin was prepared from extracts of the defatted seeds of *Croton tiglium* L. by Stillmark (1889) and had been studied in more detail by Elfstrand (1897) who noted that it was somewhat less toxic than ricin from which it differs also in respect to the specificity for the blood cells from various animals. It agglutinates red blood cells from sheep and swine but has a hemolytic action on rabbit and frog blood and is inactive on human, dog, and rat blood. Rehns (1902) stated that a crotin solution is rendered nonagglutinating and nontoxic after treatment with a suspension of erythrocytes. Karrer *et al.* (1925) reported on the purification, hemagglutinating activity, and amino acids of crotin.

A toxic protein fraction extracted from the seeds of *Jatropha curcas* has been described by Siegel (1893) who proposed the name "curcin" and considered its physiological properties to be similar to those of ricin. Mourgue *et al.* (1961) separated the seed extract from *Jatropha curcas* into eight fractions one of which was toxic. It has never been published that curcin has hemagglutinating activity.*

From the latex of another *Euphorbiacea,* the tree *Hura crepitans,* Richet (1909) obtained a protein fraction which he called crepitin and which had toxic and hemagglutinating properties similar to those of ricin.

Abrin, the toxic principle of the jequirity bean *Abrus precatorius* L.,

*See footnote *a* of Table II.

has been studied quite extensively. Warden and Waddell (1884) at Calcutta, on the occasion of a visit of R. Koch to India, prepared toxic extracts and investigated its physiological properties without observing hemagglutinating activity detected by the later investigators. Ehrlich (1891b) used antiricin and antiabrin sera to demonstrate the specific action of antitoxins. Hausmann (1902) observed the remarkable resistance of abrin to tryptic digestion. Abrin is somewhat less toxic than ricin and does not produce intestinal hemorrhages like the ricin does, but it is much more irritating to the eye (Ehrlich, 1891b).

A toxic protein has been found in the bark of the tree *Robinia pseudacacia* by Power and Cambier (1890) for which the name robin was proposed. It has properties similar to those of ricin and abrin. Ehrlich (1891b) found that an antiserum produced by the injection of subtoxic amounts of robin in rabbits will neutralize the toxicity not only of robin itself but also that of ricin. Bourrillon and Font (1968) prepared a purified hemagglutinin from *Robinia pseudacacia* by chromatographic methods. Krüpe *et al.* (1968) reported on the mitogenic activity of the crude agglutinin. Field beans or hyacinth beans, *Dolichos lablab,* are consumed in many tropical countries. The crude seeds are toxic for experimental animals and a saline extract has hemagglutinating properties. Two hemagglutinins have been prepared by Salgarkar and Sohonie (1965a) from field beans. One was homogeneous electrophoretically and the other contained three components. The major agglutinin when fed to rats in a casein diet produced growth inhibition and death in these animals (Salgarkar and Sohonie, 1965b). The growth depression caused by the purified lectin was less than that observed with an amount of field bean meal containing equivalent hemagglutinating activity.

Concanavalin A is the crystalline lectin from the jack bean, *Canavalia ensiformis,* first described by Sumner and Howell (1936). It has the unique ability to precipitate glycogen, some mucopolysaccharides, yeast mannan, and dextrans. Its amino acid composition and physical properties have been studied by Olson and Liener (1967a) who observed that a three times crystallized concanavalin A can be further purified by selective adsorption on Sephadex G-100 followed by elution with dilute acid. The finding that there was more than 1 N-terminal alanine residue present suggests a multichain structure. Subsequent work (Olson and Liener, 1967b) has revealed that concanavalin A appears to be made up of four identical subunits, each having a molecular weight of 17,500, and that dissociation into subunits can occur under certain conditions.

The intraveneous injection of concanavalin A into rabbits produces hemolysis and death (Damashek and Miller, 1943). Unheated jack bean meal may cause poor growth and death when fed to rats, although the heated seed meal has no such effect (Orrú and Demel, 1942). Shone

(1961) observed severe mucoid enteritis, nephritis, and emphysema in the lungs of cows which had died after the ingestion of jack bean meal. It has yet to be proved whether these harmful effects accompanying the ingestion of raw jack bean meal are due to concanavalin A. Mitogenic activity similar to that of bean agglutinin has been detected in concanavalin A (Wecksler *et al.* 1968).

Although an agglutinin has been isolated from potatoes by Krüpe and Ensgraber (1962) and by Marinovich (1964) and some of its chemical and physical properties were described, nothing is known about its possible activity in animals.

V. COMPOSITION

It is generally recognized that phytohemagglutinins are proteins. From the data in Table III it is evident that the physical properties vary not only between agglutinins from different species but also between those obtained from different varieties of the same plant species. Several lectins may exist in one seed sample as found for kidney beans (Jaffé and Hannig 1965; Takahashi *et al.*, 1967), field beans (Salgarkar and Sohonie, 1965a), soybeans (Lis *et al.*, 1966), and castor beans (Waller *et al.*, 1966) which can be separated because they have different solubility characteristics or behave differently in electrophoresis or chromatography and must therefore differ from each other in physicochemical properties and probably also in chemical composition. It is remarkable that despite the differences in many of the physical parameters reported in Table III for the various lectins, the molecular weights (~100,000) are similar in most cases. This is especially notable in the different bean agglutinins.

Hemagglutinins have often been classified as globulins because their electrophoretic mobility is similar to that of human serum globulin although most do not require the presence of neutral salts for solubilization. Ensgraber *et al.* (1960) compared some properties of twelve different agglutinins and found that they precipitate from aqueous solution at different concentrations of ammonium sulfate and that they differed slightly in electrophoretic mobility. The four preparations obtained in purified form had sedimentation constants between 6.5 and 7.5 S. They concluded from this study that these proteins resemble most closely the serum globulins.

No close resemblance in the patterns of the amino acids between the different legume agglutinins appears to exist. From the composition of kidney bean agglutinins reported by Jaffé and Hannig (1965), Rigas *et al.* (1966), Takahashi *et al.* (1967), and of the field bean lectins of Salgarkar and Sohonie (1965a), it is evident that all have a very low cystine con-

tent. No cystine at all was found by Olson and Liener (1967a) in concanavalin A, the agglutinin from the jack bean, and by Bourrillon and Font (1968) in the lectin from *Robinia pseudacacia*, but Huprikar and Sohonie (1965) detected 1.78% of this amino acid in the pea agglutinin and Wada *et al.* (1958) found 1.5% in the soybean lectin. Comparing the values of the amino acids from the lectin of the two plant species, kidney beans and field beans, it appears that they differ in some but not in all amino acids.

Renkonen (1950) expressed the view that the agglutinin from *Vicia cracca* is a mucoprotein but he did not identify the sugars present. Wada *et al.* (1958) detected glucosamine in soybean agglutinin, and Lis *et al.* (1964) have isolated and characterized a glycopeptide from soybean hemagglutinin which contained glucosamine and mannose. Jaffé and Hannig (1965) observed that all the seed proteins from black beans which they had studied contain sugars. In one of the hemagglutinins present in this variety of beans, they detected mannose, glucose, galactose, xylose, fucose, glucosamine, and an unidentified sugar. In another hemagglutinating fraction from the black bean, rhamnose and arabinose were also found. Takahashi *et al.* (1967) detected mannose, glucose, arabinose, galactose, fucose, xylose, and glucosamine in the wax bean agglutinin and later reported the isolation of a glycopeptide derived from this lectin (Takahashi and Liener, 1968). Bourrillon and Font (1968) prepared a glycopeptide by proteolysis of the *Robinia* agglutinin which was found to contain essentially all the agglutinating activity. The following sugars have been identified in other plant agglutinins: xylose, glucose, rhamnose, and raffinose in white peas (Huprikar and Sohonie, 1965); galactose, arabinose, xylose, and an unidentified sugar in potato agglutinin (Krüpe and Ensgraber, 1962); and xylose, fucose, mannose, and glucosamine in ricin (Jaffé *et al.*, 1964). The two lectins prepared by Salgarkar and Sohonie (1965a) from field beans also contained sugars. A notable exception appears to be the concanavalin A from jack beans in which no carbohydrate could be detected after thorough purification (Olson and Liener, 1967a).

The presence of lipids in a few phytohemagglutinins is suggested by specific staining with sudan black on paper electropherograms and immunoelectrophoresis slides observed with kidney bean agglutinin by Jaffé and Hannig (1965) and with ricin by Jaffé *et al.* (1964). The wax bean agglutinin of Takahashi *et al.* (1967) may also contain lipids because only 92.4% of its weight could be accounted for on the basis of amino acids and carbohydrates. It may be significant in this connection that Ohama (1960a,b) observed that lipase rapidly destroys the hemagglutinating action of kidney bean agglutinin and ricin.

VI. MODE OF ACTION

The pathological lesions produced in experimental animals after the injection of ricin and other toxic hemagglutinins have been the subject of numerous studies, but no explanation for the mechanism of the intoxication has yet been found. Macroscopic and microscopic lesions are similar whether ricin, abrin, crotin, or other similar toxins are injected. Most notable is the intensive inflammation with destruction of epithelial cells, edema, hyperemia, and hemorrhages in the lymphatic tissues. The liver presents fatty degeneration and necrosis, the myocard may show degenerative lesions, and the capillaries of all organs may be extended and filled with blood clots. Local hemorrhages are frequently observed at the site of injection (Brocq-Rousseu and Fabre, 1947). Changes in the quantitative composition of plasma, liver, and urine of rats acutely poisoned with ricin and a reduction of the respiratory quotient of liver slices from the same animals led Thomson (1950) to the conclusion that the toxic action of ricin may be explained by an interference with some metabolic process in the liver, possibly the Krebs cycle. Dirheimer *et al.* (1966) observed a rise of the blood values of urea, glucose, bilirubin, transaminases, and lactic dehydrogenase in ricin-poisoned rats. Albumin and hematuria could also be detected leading to the conclusion that a hepatonephritis with hepatic cytolysis may be an early manifestation of ricin intoxication. Waller *et al.* (1966) investigated the *in vitro* activity of ricin on mitochondral respiration and on the activity of crystalline fumarase without detecting any significant effect. Bálint (1967) observed that the magnesium blood level is much decreased after the injection of ricin in cats.

Little is known about the distribution of ricin in the body or its excretion. The intestinal tissue and the intestinal juice of rabbits became highly toxic after the intravenous injection of ricin indicating its concentration in this tissue and its secretion into the intestinal lumen. It could not be found in the urine (Stépanof, 1896). Ricin may be present in the milk of lactating guinea pigs which had been injected with this agglutinin after the birth of the litters because the suckling young became markedly resistant against subsequent injections of the toxin (Watson, 1905).

Application of a ricin solution by subcutaneous injection produces severe inflammation, edema, and necrosis (Madson and Walbun, 1904). Its introduction into the eye causes intensive irritation which may lead to blindness (Ehrlich 1891a). Similar reactions in the eye are produced by abrin (Ehrlich, 1891b). Schöne (1958) observed that the inflammation producing activity is not destroyed by sodium hypochlorite, but the intraperitoneal toxicity rapidly disappears when a small amount of this reagent is added to a solution of ricin.

Besides the toxic hemagglutinin there exist in castor beans several different allergenic compounds (Layton *et al.*, 1961) which may be present even in crystalline ricin (Corwin, 1961) and which may produce severe allergic reactions, asthma, and anaphylactic shock. The alkaloid from castor beans, ricinine, does not seem to have a high degree of toxicity (Murase *et al.*, 1966). The simultaneous presence of these toxicants together with ricin in castor beans makes the toxic manifestations produced by the seed products, crude extracts, and partly purified ricin preparations difficult to interpret.

A lag period between the injection of a lethal amount of ricin and the death of the injected animal of not less than 12 hr is always observed; it may be much longer if the dose is small (Corwin, 1961). Ricin is many times more toxic when injected than when given orally, but the mode of injection seems to be of little consequence (Stillmark, 1889).

Pathological lesions in animals injected with kidney bean extracts have been described by Szperl-Seyfriedowa (1951). Parenchymatous and fatty degeneration and edema are found in various tissues. Focal necrosis and fatty changes can be observed in the liver. Hemorrhages occur in the stomach, the intestinal wall, and other organs. Kidney and myocard may show distentions of capillary vessels with numerous thrombi.

Kakade *et al.* (1965) described the morphological changes in rats fed navy beans consisting of increased weight of kidney and heart, pancreatic acinar atrophy, and fatty metamorphosis of the liver. They explained these effects by the low availability of essential amino acids and low food intake of the animals consuming the raw bean diet. Multiple histological lesions were observed by Hintz *et al.* (1967) in the brains of rats fed raw kidney beans. Phadke and Sohonie (1962) found focal liver necrosis in rats fed field beans. It cannot be judged, however, to what extent these lesions were produced by the hemagglutinins existing in these seeds.

Digestibility measurements performed in rats fed a diet containing small amounts of isolated black bean agglutinin showed low food absorption and nitrogen retention in these animals (Jaffé, 1960). The absorption of glucose from a ligated intestinal loop in anesthetized rats previously fed a bean diet or given the black bean agglutinin by stomach tube was much decreased (Jaffé and Camejo, 1961). The experiments of Kakade and Evans (1966) demonstrated reduced absorption of amino acids from raw navy bean diets, an effect which may be related to the action of the hemagglutinin although other factors were not ruled out. Hintz and Hogue (1964) found raw kidney beans to interfere with vitamin E utilization in chicks (see also Chapter 13, VII,C1). The hypoglycemia observed by Hintz *et al.* (1967) in rats fed a bean diet may also be indicative of reduced intestinal absorption of glucose. When a ration contain-

ing agglutinin-free kidney beans was fed to rats, a supplement of enzyme-digested casein enhanced growth markedly, but in a diet prepared with agglutinin-containing beans this supplement had no effect at all on growth. This difference in response was attributed by Jaffé and Vega Lette (1968) to the action of the bean agglutinin on intestinal absorption.

Some observations in animals fed raw soybean diets can perhaps be explained by a similar absorption defect. Liener (1962) discussed this possibility in relation to the goitrogenic effect of soybean meal, based on the findings of Beck (1958) that fecal loss of thyroxine from the gut was larger in animals fed raw soybeans than in the controls. Absorption of fat and fatty acids in young chicks is depressed by raw soybean meal but not by soybean trypsin inhibitor (Nesheim *et al.*, 1962). Reduced utilization of vitamin D in turkeys was caused by raw soybean meal or soy protein fractions and was not observed when the heated soy products were fed (Carlson *et al.*, 1964) (see also Chapter 13,VII,B).

Lectins may be adsorbed on erythrocytes or stroma and brought into solution again by heating to 56°C. Disappearance of the agglutinating and the toxic activity from a bean lectin solution was observed when it was treated with stroma (Jaffé, 1960). At low pH no agglutination of red blood cells occurs, and adsorbed lectins dissociate from the erythrocytes as Rehns (1902) observed with ricin and Wienhaus (1909) and Rigas *et al.* (1966) observed with kidney bean agglutinin. The last named authors estimate that there are over 400,000 binding sites for kidney bean agglutinin on the surface of each erythrocyte. Ricin may be bound to many tissue cells other than the erythrocytes which can be agglutinated in this process (Rehns, 1902). Similar observations with the bean agglutinin were reported by Wienhaus (1909) and by Jaffé (1960). Steck and Wallach (1965) studied the binding of bean agglutinin on erythrocytes, lymphocytes, and mouse sarcoma cells and came to the conclusion that agglutinin adsorption and the agglutination process are related but discrete phenomena.

A hypothesis relating the hemagglutinating and toxic properties was advanced by Jaffé (1960). The reaction between the agglutinin and the cell membrane is believed to result in an alteration of the cell function thus producing the toxic effect. Only those cells bearing the specific receptor groups for the respective lectin would be affected. According to this view the reduced intestinal absorption caused by orally ingested hemagglutinins could be explained because they may combine with the cells lining the intestinal wall and thus interfere with normal activity. Evidence for an interaction between the bean lectin and intestinal cells came from the observation that the agglutinin is strongly bound when shaken with a suspension of homogenized intestinal rat tissue.

The specificity of the action of the lectins on blood cells from various animals is comparable to that of antibodies (Landsteiner, 1945). The hemagglutinating activity of many seed agglutinins can be inhibited by some sugars and oligosaccharides in a similar manner as certain hemagglutinating antibodies are inhibited (Krüpe, 1956), thus pointing to a structural relationship between these inhibitors and the receptor groups for both agglutinating agents located on the surface of the erythrocytes. Northrop and Liener (1959) studied the inhibition of the agglutinating capacity of wax bean agglutinin by some sialic acid-containing mucoproteins which are known to inhibit the agglutination of red cells by influenza and Newcastle disease virus. They found that the bean lectin was no longer able to agglutinate cells from which sialic acid had been cleaved by the action of a certain microbial enzyme, the sialidase from *Clostridium perfringens,* in much the same way as the virus loses its capacity to agglutinate sialidase-treated cells. This indicates a similarity between the hemagglutinating mechanism of the wax bean agglutinin and the virus and, at the same time, the importance of carbohydrate-containing moieties at the receptor site of the erythrocytes. The kidney bean agglutinin has a different behavior, however, because, according to Steck and Wallach (1965), mucoproteins which are active inhibitors of virus hemagglutination did not reduce the agglutinating power of that lectin.

The function of the hemagglutinin in the plant is still an open question. The intriguing similarity with the animal antibodies prompted Mitrovic and Simonovic (1959) to inject ORh^+ erythrocytes into *Begonia punctata;* they claim that extracts from these plants would agglulinate Rh^+ blood cells specifically and therefore believe that lectins may be real plant antibodies. As the phytohemagglutinins have a strong and specific affinity for certain carbohydrates, Ensgraber (1958) has suggested that they may have a function as carbohydrate fixers and may serve to store and translocate carbohydrate materials in the growing plant.

VII. DETECTION

Since phytohemagglutinins are proteins associated in the plant with other protein material, they cannot easily be detected by chemical analysis and are usually determined by their biochemical and biological activities, i.e., hemagglutination and toxicity.

For a simple agglutination test an extract is prepared by suspending 10 g of the ground material in 50 ml of 0.85% sodium chloride solution, stirring for 2 hr, and filtering. Fresh rabbit blood, obtained with an anticoagulant, is centrifuged, the plasma decanted, and the packed cells washed twice with saline solution and resuspended. A serial dilution is

performed in a row of 12 small test tubes or in a porcelain spot plate. One tube will serve as negative control. One milliliter of the red cell suspension is added to each tube and mixed. The result is read after standing for 1 hr by gentle shaking and observing any visible agglutination or adherence to the glass.

Many modifications have been proposed to make the method more sensitive and more quantitative (Kabat and Mayer, 1961). The use of ethylendiaminetetraacetic acid (EDTA) disodium salt as the anticoagulant, the omission of washing the erythrocytes, and the maintenance of the pH at 7.0 and the temperature at 20°C are the conditions recommended by Rigas *et al.* (1966). Short-time centrifugation of the tubes increases sensitivity. Goddard and Mendel (1929) recommend slanting of the tubes to increase sedimentation. A photometric method devised by Liener (1955) has proved very successful for the quantitative determination of hemagglutinating activity and has found wide acceptance. A microtechnique, originally proposed for serological investigations by Sever (1962), can be used for work with lectins also.

Miller and Boyd (1964) succeeded in locating hemagglutinins directly on electrophoretic paper strips without elution of serial sections used previously by others. The influence of such factors as temperature, time of action, and centrifugation of the red blood cell suspension on the sensitivity of the reaction after the agglutinin has been added was studied by Coulet (1954). Tobiska (1964) summarized data on the influence of different salts on the agglutination, confirming older observations of Goddard and Mendel (1929) and others that the presence of sodium chloride or some other salt is indispensable.

As shown in Table I the detection of a given hemagglutinin by the agglutination test depends on the use of the right kind of blood cells. Rabbit blood is satisfactory for the nutritionally important agglutinins but is inactive with many other lectins. The hemagglutination test may be very useful if applied correctly because it is simple and no special facilities are required. For ricin it is not as sensitive and specific as the toxicological assay since there apparently exist interfering factors in mixed feeds (Clemens, 1963). It may serve for distinguishing heated from raw castor bean press cake or pomace and for detecting grossly underheated material. For the assay of residual toxicity it should be supplemented by animal tests. The microscopic examination for the detection of castor bean meal to unsatisfactory as heated and nonheated material cannot be distinguished.

For parenteral toxicity tests mice or rats are mostly used. Feeding experiments have been performed with a wide variety of animals, but rats and chicks are often preferred because of the ease of handling and

for economic reasons. Mourgue *et al.* (1958) have used successfully a small fish, *Gamburia holbrooki*, for the evaluation of castor bean extracts and fractions, and Janssen (1964) used another fish, *Platypoecilus variatus*.

In the feeding test, growth retardation and mortality as compared to appropriate controls are taken as measures of toxicity. When the test substance is injected, the percentage of animals which die and the time between injection and death are useful parameters for the estimation of the toxicity. Several different doses should be used and expressed as μg/kg of body weight. As susceptibility may vary considerably with weight and age the animals must be selected accordingly.

Clarke (1953) proposed the following specific test for ricin: 10 g of the finely ground material is twice extracted with 50 ml of 0.02 *N* HCl for several hours with mechanical stirring. The extract is centrifuged and 300 ml of acetone added. The precipitate is separated by filtering through sintered glass, dissolved in 5 ml of physiological saline, and filtered. Two series of identical dilutions are prepared, 0.1 ml of normal serum is added to each tube of one dilution series and 0.1 ml of serum from a rabbit immunized with ricin to each of the other series. A suitable number of mice are injected with an equal amount of the mixtures of each tube. The mice injected with the extract of the first series will have higher mortality than those of the second. Usually 1 ppm of ricin can be detected, but in a mixture with linseed meal about 0.1% has been found to be the lower limit of detection.

A skin test in guinea pigs, based on the fact that subcutaneous injections of sublethal doses produce edema, had been used in early investigations on the toxin-antitoxin reaction by Madson and Walbum (1904).

VII. DETOXIFICATION AND SIGNIFICANCE

There exist of course a great number of methods for the denaturation of proteins which probably will inactivate the phytohemagglutinins. Some have been studied for reasons of interest in the reaction mechanism or supression of specific activity (Liener, 1958a; Mourgue *et al.*, 1958). Practical application is limited to heat treatment. The destruction of the poisonous action of ricin by heat was already observed by Stillmark (1889). The agglutinating and toxic action of legumes probably had not been discovered earlier because of its disappearance after cooking.

It is therefore of considerable practical importance to know the exact conditions of heating which secure complete destruction of the toxic action with a minimum of heat damage to the proteins (Liener, 1958b). The presence of more than one toxic principle which differ in heat re-

sistance in castor beans must be taken into account when working with products derived from these seeds. The castor bean allergen* is more resistant to boiling than is ricin but is inactivated by autoclaving (Jenkins, 1963). The detoxification of castor pomace is essential for its safe handling as fertilizer and its utilization in animal feeding. Steam heating, as used for the recovery of solvents employed for the extraction of the castor oil, has been found to produce a thousandfold reduction in toxicity and to render the pomace harmless for sheep, rabbits, and rats when used in the respective diets in a proportion of not more than 10% (Clemens, 1963). Jenkins (1963) used 1-hr steam heating at 15 lb/in V^2 and found that the toxicity was reduced to about 1/2000 of its original value. Rats fed 23.9% of the autoclaved castor bean meal in a casein diet were in good health after 4 weeks, but growth and food conversion was lower than in the controls. Autoclaving in the presence of 2% NaOH, dry heating at 205°C, or moist cooking with 2% NaOH and 10% formaldehyde destroyed the allergenic action together with the ricin (Gardner *et al.*, 1960). Calcium hydroxide has also been used as an additive to castor bean residues before heating to secure complete destruction of the ricin and the allergen (Spies *et al.*, 1963). The amount of water-soluble albumin remaining after heat treatment, which can be determined turbidimetrically, is approximately proportional to the detoxification (Funatsu *et al.*, 1963). Autoclaving and subsequent extraction with water for the elimination of ricinine has been proposed for castor bean press cakes to be used in chick rations, although the toxicity of this alkaloid in the amounts found in the cake is low (Okamoto *et al.*, 1965).

Cornevin (1897) immunized animals by injecting them with heated ricin solution. They became resistant to the ingestion of castor bean cake, and the method was proposed for practical application. Mice are protected against the fatal action of ricin by the injection of blood serum from immunized goats if given not later than 6 hr after the ricin injection (Clarke and Jackson, 1956).

The nutritive value of many legumes is enhanced by autoclaving, and this effect is probably related to the destruction of toxic hemagglutinins and other growth inhibiting factors. Preliminary soaking prior to autoclaving is required for complete elimination of the toxicity of kidney bean (Jaffé, 1949) and field beans (Phadke and Sohonie, 1962). Kakade and Evans (1965b) found that autoclaving for 5 min was sufficient to eliminate the toxicity of finely ground navy bean meal. Thirty min of dry heating had little effect on hemagglutinating activity of certain varieties of *P. vulgaris*, and activity was still detectable after 18 hr of heating (de Muelenaere, 1964). Osborne and Mendel (1917) had already noted

*The allergenicity of castor bean is discussed in Chapter 11,V,E.

that dry heat is less effective than cooking for the improvement of the growth promoting action in soybeans, and Heintze (1950) found dry heat or high frequency treatment ineffective, but heating of the soaked beans or autoclaving was fully effective. Germination of soybeans improves the nutritional value (Mattingly and Bird, 1945), but in field beans no such effect was observed (Phadke and Sohonie, 1962).

Formaldehyde reduces the agglutinating and toxic actions of ricin and the agglutinating activity of bean lectin, but phenol was inactive in this respect. Potato lectin was more rapidly destroyed by phenol than by formaldehyde. Formaldehyde-inactivated ricin was still capable of inducing immunization (Ohya, 1929).

Instances of poisoning due to the ingestion of castor beans taken as a purgative were relatively frequent until the last century. Stillmark (1889) lists 112 cases of accidental poisoning by castor beans or castor products, eight of them fatal. When the toxic properties of ricin became known these accidents became very rare. Cases of children who swallow ricinus seeds and suffer the fatal consequences still occur occasionaly (Astalf, 1963). Modern methods of castor oil production exclude the contamination of this product with the toxic protein.

Of more serious concern are the hazards of handling the large amounts of press cake or pomace, a byproduct of the castor oil industry which is mainly used as fertilizer (Bolley and Holmes, 1958) since it is a good source of nitrogen, phosphorous, and potassium and has good nitrificating qualities (Naik *et al.*, 1961). Persons handling castor bean pomace which had not been submitted to the usual heat treatment have developed symptoms of irritation of eyes, nose, and throat, asthma, nausea, vomiting, weakness, and pain. Although most of these symptoms are manifestations of the allergenicity of castor bean (see Chapter 11,V,E), Cooper *et al.*, (1964) attribute at least part of these symptoms to the ricin.

The outbreak of massive poisoning after the consumption of partially cooked bean flakes has been reported by Griebel (1950). Human cases of intoxication by runner beans have been observed by Faschingbauer and Kofler (1929). The addition of kidney bean flour to wheat flour destined for the manufacture of bread (Anonymous, 1948) and the use of field bean flour for the production of bean cakes (Marcos and Boctor, 1959) have been proposed, but the utilization of these and other legumes known to contain toxic agglutinins for foods exposed to dry heating and not to cooking should be viewed with caution.

IX. FUTURE OUTLOOK

The number of papers published on plant agglutinins during the last century and the first quarter of the present is large compared to those

which appeared thereafter. Many of the older original observations have not been confirmed with modern laboratory methods. This demonstrates the decline of interest in this field.

Biological investigations during the last century and the first decades of the present were mostly concerned with describing phenomena which could not yet be explained. This situation has changed especially in biochemistry with the recent advances in enzymology, molecular biology, etc. Most workers therefore prefer these areas at the expense of others in which the breakthrough to the explanation of the observed facts has not yet been achieved. There are certainly important discoveries to be expected in the biochemical interpretation of the action of the agglutinins and their toxic properties which may have interesting implications in other fields. The specificity of action of the lectins is already a useful tool for the study of receptor groups in erythrocytes and probably can be applied to other tissues as well. As models for the antigen-antibody reaction they may still yield further important results. A full understanding of the toxic actions and their physiopathological interpretation would be highly desirable for theoretical and practical reasons. Therefore it is to be hoped that this area of research will attract more investigators in the future.

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