considered dangerous, possibly causing permanent brain damage, and their use is illegal.

CONCLUSIONS

In the past several decades great strides have been made in man's understanding of his physiological mechanisms. During this time an increasing awareness of the potential of chemical substances to alter physiologic and psychologic states has emerged. While this awareness has provided the basis for development of pharmaceutical agents to treat disease, it has also led to experimentation with potentially dangerous drugs by the general population. It is clear that many compounds which are greatly beneficial to man when prescribed under controlled conditions are very dangerous when consumed in unknown quantities in plant or food substances. In addition to direct toxicity, we have the problem that food substances not toxic to untreated humans may become toxic when eaten by individuals receiving certain pharmaceutical agents. A classic example of this situation is the toxicity of cheese containing substantial amounts of tyramine as discussed above. An increasingly important aspect of drug evaluation, therefore, is knowing if the compound interacts with other drugs or with constituents of common foods.

LITERATURE CITED

Asatoor, A. M., Levi, A. J., Milne, M. D., *Lancet* **2**, 733 (1963).
Blackwell, B., Marley, E., Mabbitt, L. A., *Lancet* **1**, 940 (1965).
Blackwell, B., Marley, E., Price, J., Taylor, D., *Brit. J. Psychiat.* **113**, 349 (1967).
Fish, M. S., Horning, E. C., *J. Nerv. Ment. Dis.* **124**, 33 (1956).
Foy, J. M., Parratt, J. R., *J. Pharm. Pharmacol.* **12**, 360 (1960).
Foy, J. M., Parratt, J. R., *J. Pharm. Pharmacol.* **13**, 382 (1961).
Foy, J. M., Parratt, J. R., *Lancet* **1**, 942 (1962).
Goth, A., "Medical Pharmacology," Mosby, C. V., Ed., St. Louis, Mo., 1968, p 335.
Green, R. C., *Vir. Med. Month.* **86**, 386 (1959).
Hoffer, A., Osmond, H., "The Hallucinogens," Academic Press, New York, N. Y., 1967, p 211.
Hofmann, A., "Drugs Affecting the Central Nervous System," Vol. 2, Burger, A., Ed., Marcel Dekker, 1962, p 169.
Horwitz, D., Lovenberg, W., Engelman, K., Sjoerdsma, A., *J. Amer. Med. Ass.* **188**, 1108 (1964).
Ingram, A. L., *J. Amer. Med. Ass.* **190**, 1133 (1964).
Jackson, B., Reed, A., *J. Amer. Med. Ass.* **207** 1349 (1969).
Jacobziner, H., Raybin, H. W., *N. Y. State Med. J.* **61**, 301 (1961).
Lovenberg, W., "Toxicants Occurring Naturally in Foods," Strong, F. M., Ed., National Research Council, 1973, in press.
McElvain, S. M., Eisenbraun, E. J., *J. Amer. Chem. Soc.* **77**, 1599 (1955).
Pinder, A. R., *Nature (London)* **168**, 1090 (1951).
Sen, N. P., *J. Food Sci.* **34**, 22 (1969).
Sjoerdsma, A., "Cecil-Loeb Textbook of Medicine," 11th ed., Beeson, P. B., McDermott, W., Ed., W. B. Saunders, Philadelphia, 1963, p 1447.
Stromberg, V. L., *J. Amer. Chem. Soc.* **76**, 1707 (1954).
Udenfriend, S., Lovenberg, W., Sjoerdsma, A., *Arch. Biochem. Biophys.* **85**, 487 (1959).
Wheaton, T. A., Stewart, I., *Anal. Biochem.* **12**, 585 (1965).
Wheaton, T. A., Stewart, I., *Phytochemistry* **8**, 85 (1969).
Weiss, G., *Psychiat. Quart.* **34**, 346 (1960).

Received for review April 10, 1973. Accepted October 4, 1973. Presented at the Symposium on Toxic Proteins, Peptides, and Related Substances, 165th National Meeting of the American Chemical Society, Dallas, Texas, April 1973.

Toxic Proteins Produced by *Clostridium botulinum*

Edward J. Schantz* and H. Sugiyama

The chemical, physical, and biological properties of *Clostridium botulinum* toxins are reviewed. Highly purified toxins in acidic solutions behave as simple proteins with molecular weights 200,000 to 900,000. These proteins are the most toxic substances known and cause botulism in man and animals. As found in culture, the molecule is a complex of at least two simple proteins: one the neurotoxin, molecular weight about 150,000, and the other nontoxic of high molecular weight. The complex is produced in foods under anaerobic conditions. It is quite stable under acidic conditions at room temperature. Alkaline conditions cause dissociation of the proteins. The separated neurotoxin is much less stable, particularly to the digestive enzymes. All of these toxins are readily destroyed by heating to 100°. They are good antigens and can be toxoided for safe immunization of man and animals.

The spore-forming bacterium, *Clostridium botulinum*, found in many soils throughout the world, produces a series of toxins that have caused food poisoning in man and animals from time immemorial. The poisoning, now called botulism, was not recognized as being caused by a food-borne microbial toxin until the discoveries of Professor Van Ermengem of the University of Ghent about the end of the 19th Century (Dolman, 1964). Eight immunologically distinct toxins are now recognized and are designated types A, B, C_1, C_2, D, E, F, and G. As far as we know, all of these toxins are simple proteins and the most toxic substances known to man. The organism normally produces the toxins in foods that are canned or preserved under anaerobic conditions. Generally, a strain of *C. botulinum* produces one toxin and is classified according to the toxin it produces. The types of toxins and the organisms producing them are illustrated in Table I.

Toxin types A, B, E, and to some extent F, have caused most of the cases of botulism in man, and types C_1, C_2, D, and E have caused most of the cases of botulism in the domestic and wild animal populations of the world. Botulism from types A and B in the United States has usually resulted from contaminated canned vegetables and fruits. In Europe contaminated meat and fish products are relatively more common causes of botulism, and in Japan and some parts of Russia practically all cases are from contaminated fish products. Type E botulism occurs mainly from contaminated foods of marine origin, but fresh water fish have been involved also. This particular type of organism is found along many marine coasts of the world and in the Great Lakes of the United States. About 90% of all cases of botulism in man have resulted from the toxin formed in improperly sterilized home-processed

Food Research Institute and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706.

Table I. Toxic Proteins Produced by *Clostridium botulinum*

C. botulinum type	Toxins produced: Major type	Toxins produced: Minor type	Species principally effected
A	A	F (one strain)	Man
B	B		Man
C_α	C_1	C_2, D	Water fowl
C_β	C_2		Cattle and horses
D	D	C_1, C_2	Cattle
E	E		Man and animals
F	F		Man (two outbreaks)
G	G		Unknown[a]

[a] Type G has been isolated from soil. As far as is known it has not caused cases of botulism in man or animals under natural conditions.

foods. Outbreaks of botulism from commercially canned foods have been much less frequent. They usually result from improper sterilization due to accidental malfunction of processing equipment or from changes to processing procedures which have not been adequately evaluated.

Clinical Picture. The first symptoms may be vomiting and other gastrointestinal disturbances within 12 to 36 hr after ingesting the toxin. A lassitude and weakness develop and this is followed by dryness of the mouth and throat, with difficulty in swallowing. Indications of paralysis appear, including double vision, drooping of the facial muscles, weakness of the tongue, diaphragm, neck, and extremities. Respiratory distress becomes progressively more marked and is the main cause of death, although airway obstruction, pulmonary infection, and cardiac arrest may be involved. Effects may vary from one individual to another and vary some for the different types of toxin. The amount of toxin to cause death in man has been estimated from accidental cases of botulism to be between 0.1 and 1 μg or 3000 to 30,000 mouse intraperitoneal LD_{50}.

Tests for Botulinum Toxin. The most sensitive and reliable test for botulinum toxin in foods, blood, and stomach contents is the test in mice. A small quantity (0.5 ml is recommended) of the liquid from the sample or an extract of it made with 0.05 *M* phosphate buffer at pH 6.8 is injected intraperitoneally into white mice weighing about 20 g. If the mice die within 96 hr the killing agent must be identified. This can be accomplished by treating separate aliquots of the sample with antisera to the different toxin types. The type of antitoxin that neutralizes the toxic agent establishes the type of toxin in the sample. Quantitation of the toxin can be done by preparing serial dilutions of the sample and injecting these into separate groups of mice containing six or eight mice in each group. The percent kill in each group is plotted against the dose on probit-log dose paper. The best straight line is fitted by inspection and the dose corresponding to probit 5 is read off the graph as the LD_{50} dose. The mouse data also can be treated by procedures such as the Reed and Muench (1938) calculations. For the strain of white mice used in this laboratory, one LD_{50} of type A toxin is equivalent to 3×10^{-5} μg.

Tests for the toxin based on the immunological properties have been reported by several investigators (Johnson *et al.*, 1966; Rycaj, 1956; Uemura and Sakaguchi, 1971). These tests have some advantages over the mouse assay in that the reagents (antitoxin and red blood cells) can be kept on hand for use at any time, whereas mice of the proper age and weight may not be available when needed. Evancho *et al.* (1973) have reported a standardized reversed passive hemagglutination technique that is applicable to foods. Antibody to a particular type of toxin is adsorbed on red blood cells. When these sensitized cells come in contact with the same type of toxin, agglutination of the cells occurs. Since toxoid reacts approximately the same as the toxin it is used as the standard of comparison, making it unnecessary to distribute and handle toxin. In this particular technique the highest dilution of the unknown that gives a hemagglutination end point is considered to have the same amount of toxin as the smallest amount of toxoid that gives a hemagglutination end point. The procedure will detect 0.0008 μg of crystalline type A toxin (produced by Hall strain) per milliliter or about 25 mouse LD_{50}.

Table II. Sedimentation Rates of Different Types of Botulinum Toxin in Culture Filtrates

Type	LD_{50}/ml,[a] original culture	LD_{50}/ml,[a] centrifuged culture	s (20°), sec $\times 10^{13}$
A	1.1	0.3	19
B	0.30	0.14	16
C	0.10	0.05	14
D	0.80	0.38	16
E	0.1	0.05	14
F	0.14	0.07	14

Fresh culture was centrifuged for 90 min at 39,000 rpm in a Spinco preparative ultracentrifuge as described by Schantz (1967). The temperature was 4° and was corrected to 20°.

Column 2 shows the toxicity of the original culture and column 3 shows the toxicity of the top 1 cm of the tube in a SW 39 rotor.

[a] LD_{50} in millions.

Purification and Properties of the Toxins. Lamanna and coworkers (1946) and Abrams *et al.* (1946) at Fort Detrick were the first to isolate type A toxin in crystalline form. The purification was accomplished by Lamanna *et al.* by precipitation of the toxin from the culture with acid at pH 3.5, shaking with chloroform and crystallization from ammonium sulfate solutions and by Abrams *et al.* by repeated precipitation with sodium sulfate. Duff *et al.* (1957) improved the method by substituting precipitation with alcohol at −5° for the shaking with chloroform. The crystalline type A proved to be a simple protein, composed of amino acids only, with a high molecular weight of 900,000 (Buehler *et al.*, 1947; Kegeles, 1946; Putnam *et al.*, 1946; Stefanye *et al.*, 1967). The crystals are white needles usually about 100 μ in length and, when dissolved in aqueous buffers at pH 4.3, act like homogenous substances in ultracentrifugation and in electrophoresis. The toxin has the solubility characteristics of a globulin and has an isoelectric point at pH 5.6. The Fort Detrick workers also were successful in obtaining relatively pure types B, C, D, and E by employing the above techniques (Cardella *et al.*, 1958, 1960; Gordon *et al.*, 1957; Lamanna and Glasman, 1947). Later Sakaguchi and Sakaguchi (1961) in Japan purified type E toxin having a molecular weight of 350,000 by chromatographic procedures. In their studies of what they called the ultimate toxic unit, Gerwing and coworkers (1965a,b, 1966a) reported the purification by chromatography on DEAE cellulose of type A toxin of molecular weight of 12,000, type B toxin of a molecular weight of 10,000, and type E toxin of molecular weight 18,000. Several investigators have been unable to repeat the work of Gerwing and coworkers but these reports stimulated further investigations on the molecular size of these toxins. Schantz (1967) studied the sedimentation rate of toxin types A, B, C, D, E, and F in undiluted culture (pH about 5.6), using the bioassay with mice to follow the distribution of the toxin, and found the rates to be relatively fast and in line with proteins having molecular weights of 200,000 to 900,000. These results are shown in Table II. It is assumed therefore that the high molecular weight toxins which have been isolated are not artifacts of the purification procedures.

After obtaining type A toxin as crystals, Lamanna found that it was a complex of at least two proteins, one the toxin and the other a nontoxic protein with hemaggluti-

nating property (Lamanna and Lowenthal, 1951). When a solution of the toxin of slightly alkaline pH was treated with red blood cells, hemagglutination occurred. The supernatant fluid no longer had hemagglutinating properties, but had all of the toxicity. Studies by Wagman and Bateman (1953) and Wagman (1954) showed that crystalline A toxin dissolved in a buffer of pH more acidic than its isoelectric point was homogenous during ultracentrifugation but at a pH of 6.8 or above and at an ionic concentration of 0.13 or more, the toxin dissociated into two components. One component had a molecular weight of about 500,000 and possessed the hemagglutinating properties, while the other had an average molecular weight of 70,000 and possessed two to three times the specific toxicity of the parent toxin. In line with these results, DasGupta and Boroff (1967) fractionated the crystalline type A toxin on columns of DEAE cellulose and obtained a hemagglutinating fraction of high molecular weight and a toxic fraction of molecular weight of 120,000 to 150,000, depending upon the method of determination. The toxic fraction had three to five times the specific toxicity of the parent toxin. These workers considered the smaller protein, constituting about 20% of the crystalline toxin, to be the neurotoxin and designated it the α fraction. The hemagglutinating fraction (about 80%) was designated the β fraction. Knox *et al.* (1969), using similar techniques, obtained results in line with those of the above investigators. DasGupta *et al.* (1968) and Beers and Reich (1969) have isolated a type B neurotoxin of 167,000 molecular weight. When its associated nontoxic protein is removed, type E neurotoxin is of molecular weight 150,000 (Kitamura *et al.* 1969). Type F toxin having a molecular weight of 150,000 has been purified to a high degree by Yang and Sugiyama (unpublished results).

It appears that the organism does not produce the fully toxic molecule, but rather a progenitor toxin that is activated to its full toxicity by certain enzymes produced in the culture. Type E (Sakaguchi and Tohyama, 1955) and nonproteolytic strains of other types apparently do not produce such progenitor activating enzymes. Duff *et al.* (1956) found that this toxic form (progenitor) could be converted to its full toxicity by treatment with trypsin. Conversion of the type E progenitor toxin to its full potency can increase the toxicity as high as 100-fold or more. Bonventre and Kemp (1959) have pointed out that the proteolytic organisms that produce types A and B toxins also produce progenitor toxins, but these organisms produce proteinase(s) that activate the toxins to their full potential toxicity. DasGupta and Sugiyama (1972a) have purified one of these enzymes and have shown it to have trypsin-like properties. In regard to the size of the activated molecule, Kitamura *et al.* (1969) treated type E toxin with trypsin and found that the molecular weight did not change on activation. DasGupta and Sugiyama (1972b) have confirmed the results of Sakaguchi and Sakaguchi (1961) with type E progenitor neurotoxin of a molecular weight 150,000. They also found no change in the molecular weight upon activation with trypsin, but when the trypsinized toxin was treated additionally with a disulfide reducing agent (β-mercaptoethanol) and sodium dodecyl sulfate before being subjected to polyacrylamide gel electrophoresis, the neurotoxin was further split into two fractions of about 50,000 and 100,000 molecular weights. Apparently, at least one peptide bond is split during the activation, but the two fractions are still held together by a disulfide bond(s) whose reduction permits the separation of the two fractions.

The nontoxic component of the toxin complex appears to make the neurotoxin more stable. The neurotoxin by itself loses toxicity unless it is held in the presence of some protein such as gelatin or the blood serum proteins. It is particularly labile to some enzymes of the digestive tract and for that reason it is likely that it would not cause botulism in man if it occurred in his food. Several thousand times more neurotoxin than the toxin complex is required to cause botulism in animals by feeding. Boroff *et al.* (1972) have recently proposed, on the basis of electron micrographs, that the hemagglutinin portion of the type A molecule forms a shield around the neurotoxin that protects it against adverse effects. The means by which the neurotoxin is bound to the nontoxin portion of the toxin complex is not understood. Whatever the nature of the bonding, it apparently provides protection to allow some of the neurotoxin to survive the digestive processes and get into the blood stream. Pepsin and trypsin slowly destroy the toxicity of crystalline type A toxin but much more slowly than other proteins are hydrolyzed by these enzymes. The acidic condition of the stomach would tend to keep the complex bound together, and the alkaline conditions encountered later in the intestine would favor dissociation into units that could be absorbed into the blood stream.

The neurotoxin of about 150,000 molecular weight may dissociate or be broken into smaller toxic units. Wagman (1963) reported a dialyzable toxic unit as small as 3800 in a pepsin digest of the toxin previously treated with alkali at about pH 9. Schantz and Lauffer (1962) reported the isolation of a unit between 20,000 and 40,000, as indicated by diffusion measurements in agar gel buffered with 0.1 *M* sodium phosphate at pH 7.3 and in the presence of 0.1% gelatin to stabilize the neurotoxin.

A small toxin molecule would help explain how the toxin passed through the various membranes of the body to the site of action. Perhaps a small portion of the molecule, such as a prosthetic group of some amino acids, is the ultimate unit of toxin. It could be small enough to be dialyzable and pass from one protein to another in the body until it attached to the site of action. Heckley *et al.* (1960) and Hildebrand *et al.* (1961) found that the toxin present in the blood of poisoned animals was much smaller than the crystalline type A toxin and in line with the dimensions of the α or neurotoxin fraction.

The crystalline type A toxin is readily surface denatured in stretching thin films such as that formed when bubbling air or other gases through a solution of the toxin. This denaturation takes place when air, nitrogen, or carbon dioxide is bubbled through the solution of the toxin in acetate buffer at pH 4.2 and in phosphate buffer at pH 6.8. Schantz *et al.* (1960) found that the toxin also was readily detoxified in 6 *M* urea and 3 *M* guanidine solutions and that the toxicity was not restored on removal of the urea or guanidine. These facts strongly indicate that the toxicity must be due to the conformational structure of the molecule. Schantz and Spero (1956) acetylated the free amino groups with ketene and found that a rapid detoxification took place. When 5% of the amino groups was acetylated, as indicated by the Van Slyke method, 43% of the toxicity was lost, and when 19% of the groups was acetylated, 98% of the toxicity was lost. Deamination with nitrous acid resulted in a similar loss of the toxicity (Spero and Schantz, 1956). A reasonable assumption from these data is that a few of the amino groups are quite vulnerable to some outside effects, and these groups also are critical in holding the molecule in the shape required for its toxic activity. It appears too that the sulfhydryl groups are involved in holding the molecule in the toxic shape. Treatment of the toxin with *p*-mercuribenzoate (PMB) causes some loss in toxicity, but at a much slower rate than that caused by acetylation of the amino groups. Gerwing *et al.* (1966–1967) reported that the neurotoxin units (mol wt 10,000 to 18,000) of the toxin from types A, B, and E were over 90% inactivated by PMB and *n*-substituted maleimide and suggested that a cysteinyl residue played a critical role in the toxicity. Both crystalline toxin A (mol wt 900,000) and the neurotoxin (mol wt 150,000) derived from it have four -SH groups and one disulfide

bridge per 100,000 molecular weight. Beers and Reich (1969) found that PMB and iodoacetate inactivated the neurotoxic unit of type B toxin. Knox *et al.* (1970) studied this reaction and reported that both the crystalline type A and the neurotoxin were 90% inactivated by PMB in a 24-hr period. Iodoacetamide caused a smaller reduction but *N*-ethylmaleimide had no effect. Reversal of the action of PMB could not be accomplished with reduced glutathione. These workers concluded that -SH groups may affect the conformational stability, but these groups are not an integral part of the active center. Sugiyama *et al.* (1973) have found that dithiothreitol, a disulfide reducing agent, destroys practically all of the toxicity within 30 min at 37°.

If toxicity is a function of shape, other reactions that break bonds or modify the structure should also cause detoxification. Weil *et al.* (1957) irradiated type A toxin in the presence of methylene blue, a reaction that modifies the amino acid histidine, and found a rapid detoxification. Boroff and DasGupta (1964) applied this technique under a different set of conditions that modifies the amino acid tryptophane and also found a rapid detoxification. On the basis of these results, the latter investigators proposed that tryptophane is the active center of toxicity in the toxin molecule. At this stage of our knowledge of the toxin molecule, it seems quite speculative to assign the specific action to any one amino acid. It appears more likely that the detoxification could be accomplished by breaking of a bond or the modifying of an amino acid that was involved in the holding of the toxin in its toxic shape. The actual site of toxic activity of the toxins appears far from settled.

All botulinal toxin types are readily destroyed by heat at relatively low temperatures. The inactivation rate depends to a great extent on the pH of the medium containing the toxin, the more alkaline, the faster the detoxification. Cartwright and Lauffer (1958) found that solutions of the crystalline type A toxin can be heated at 40° at pH 6.9 for 1 hr without loss of activity, but at 50° less than 1% is active after 10 min, and at 100° detoxification occurs instantly. At 15° and at pH 10, Spero (1958) found that the toxicity remained stable for 3 hr, but at pH 10.8 over 50% was detoxified within an hour, and at pH 11, 99.9% was detoxified in 1 hr.

Crystalline type A toxin can be preserved for a year or more by dissolving it in 0.05 *M* acetate buffer at pH 4.2 and keeping it at about 4°. However, solutions of the toxin in acetate buffer must not be frozen or complete loss of toxicity will occur (Stefanye *et al.*, 1964). Another means of preserving the crystalline toxin is to dissolve it in 0.05 *M* phosphate buffer at pH 6.8 and freeze-dry it. These preparations have been kept several years with no loss in toxicity.

Biological Action of Botulinum Toxins. The exact mechanism by which the toxins cause paralysis is not fully understood. The toxin inhibits cholinergic synaptic transmission from one nerve to another and from nerve endings to effector elements. It is believed, on the basis of studies by Brooks (1964), that the block is accomplished by interfering with some as yet unspecified step in the release of acetylcholine from the nerve endings. It is hoped that the use of purified toxins for studies on the mechanism of action of the toxins may lead to a means of treatment of botulism more effective than is now available. The only specific treatment at present is the administration of antitoxin and artificial respiration. Except for type E toxin, the administration of antisera is not very effective after symptoms have appeared (Koenig, 1971). What is needed is something that will reverse the action at the sites where the toxin is bound or something that will stimulate the blocked synapse to function until the natural recovery has been accomplished. Cherington and Ryan (1968) has reported some relief in animals and man by treatment with guanidine, a substance that accelerates the release of acetylcholine at the nerve endings. However, these studies are in an experimental stage and not conclusive.

The toxins are good antigens, but natural immunity in people rarely occurs if at all because the dose to cause death is less than that to elicit antibody production. The toxins can be toxoided with formaldehyde, resulting in the complete destruction of the toxicity and retention of antigenicity to effectively immunize man and animals. Besides the production of antisera for medical treatment of botulism in man, the toxoids are valuable for the immunization of people that work with the toxin, livestock, and some fur-bearing animals against botulism.

LITERATURE CITED

Abrams, A., Kegeles, G., Hottle, G. A., *J. Biol. Chem.* **164,** (1946).
Beers, W. H., Reich, E., *J. Biol. Chem.* **244,** 4473 (1969).
Bonventre, P. F., Kemp, L. L., *J. Bacteriol.* **78,** 892 (1959).
Boroff, D. A., DasGupta, B. R., *J. Biol. Chem.* **239,** 3694 (1964).
Boroff, D. A., Sverker, N., Hoglund, S., *Infect. Immunol.* **6,** 1003 (1972).
Brooks, V. B., United States Public Health Service Publication No. 999-FP-1, 1964.
Buehler, H. J., Schantz, E. J., Lamanna, C., *J. Biol. Chem.* **169,** 295 (1947).
Cardella, M. A., Duff, J. T., Gottfried, C., Begel, J. S., *J. Bacteriol.* **75,** 360 (1958).
Cardella, M. A., Duff, J. T., Wingfield, B. H., Gottfried, C., *J. Bacteriol.* **79,** 372 (1960).
Cartwright, T. E., Lauffer, M. A., *Proc. Soc. Exp. Biol. Med.* **98,** 327 (1958).
Cherington, M., Ryan, D. W., *N. Engl. J. Med.* **278,** 931 (1968).
DasGupta, B. R., Boroff, D., *Biochem. Biophys. Acta* **147,** 603 (1967).
DasGupta, B. R., Boroff, D. A., Rothstein, E., *Biochem. Biophys. Res. Commun.* **32,** 1057 (1968).
DasGupta, B. R., Sugiyama, H., *Biochem. Biophys. Acta* **268,** 719 (1972a).
DasGupta, B. R., Sugiyama, H., *Biochem. Biophys. Res. Commun.* **48,** 108 (1972b).
Dolman, C. E., Public Health Service Publication 999-FP-1, 1964, p 5.
Duff, J. T., Wright, G. G., Klerer, J., Moore, D. E., Bibler, R. H., *J. Bacteriol.* **73,** 42 (1957).
Duff, J. T., Wright, G. G., Yarinsky, A., *J. Bacteriol.* **72,** 455 (1956).
Evancho, G. M., Ashton, D. H., Briskey, E. J., Schantz, E. J., *J. Food Sci.* **38,** 764 (1973).
Gerwing, J., Dolman, C. E., Bains, H. S., *J. Bacteriol.* **89,** 1383 (1965a).
Gerwing, J., Dolman, C. E., Kason, D. V., Tremaine, J. H., *J. Bacteriol.* **91,** 484 (1966a).
Gerwing, J., Dolman, C. E., Reichman, M. E., Bains, H. S., *J. Bacteriol.* **89,** 1176 (1965b).
Gerwing, J., Ko, A., Van Alystyne, D., Tremaine, J. H., *Biochem. Biophys. Acta* **117,** 487 (1966b).
Gerwing, J., Mitchell, B., Van Alystyne, D., *Biochem. Biophys. Acta* **140,** 363 (1967).
Gordon, M., Fiock, M. A., Yarnisky, A., Duff, J. T., *J. Bacteriol.* **74,** 533 (1957).
Heckley, R. J., Hildebrand, G. J., Lamanna, C., *J. Exp. Med.* **111,** 745 (1960).
Hildebrand, G. J., Lamanna, C., Heckley, R. J., *Proc. Soc. Exp. Biol. Med.* **107,** 284 (1961).
Johnson, H. M., Brenner, K., Angelotti, R., Hall, H. E., *J. Bacteriol.* **91,** 967 (1966).
Kegeles, G., *J. Amer. Chem. Soc.* **68,** 1670 (1946).
Kitamura, M. S., Sakaguchi, S., Sakaguchi, G., *J. Bacteriol.* **98,** 1173 (1969).
Knox, J. N., Brown, W. P., Spero, L., *Infect. Immunol.* **1,** 205 (1969).
Knox, J. N., Brown, W. P., Spero, L., *Biochem. Biophys. Acta* **214,** 350 (1970).
Koenig, M. G., "Neuropoisons. Vol. 1. Poisons of Animal Origin," Simpson, L. L., Ed., Plenum Press, New York, N. Y., 283 1971, p 283.
Lamanna, C., Eklund, H. W., McElroy, D. E., *Science* **103,** 613 (1946).
Lamanna, C., Glasman, H. N., *J. Bacteriol.* **54,** 575 (1947).
Lamanna, C., Lowenthal, J. P., *J. Bacteriol.* **61,** 751 (1951).
Putnam, F. W., Lamanna, C., Sharp, D. G., *J. Biol. Chem.* **165,** 735 (1946).
Reed, L. J., Muench, H., *Amer. J. Hyg.* **27,** 493 (1938).
Rycaj, T., *Bull. Acad. Pol. Sci. Cl.* 2 **4,** 317 (1956).

Sakaguchi, G., Sakaguchi, S., *Jap. J. Med. Sci. Biol.* **14,** 242 (1961).
Sakaguchi, G., Tohyama, Y., *Jap. J. Med. Sci. Biol.* **8,** 255 (1955).
Schantz, E. J., *Jap. J. Microbiol.* **11,** 380 (1967).
Schantz, E. J., Lauffer, M. A., *Biochemistry* **1,** 658 (1962).
Schantz, E. J., Spero, L., *J. Amer. Chem. Soc.* **79,** 1623 (1956).
Schantz, E. J., Stefanye, D., Spero, L., *J. Biol. Chem.* **235,** 3489 (1960).
Spero, L., *Arch. Biochem. Biophys.* **73,** 484 (1958).
Spero, L., Schantz, E. J., *J. Amer. Chem. Soc.* **79,** 1625 (1956).
Stefanye, D., Iwamasa, R. T., Schantz, E. J., Spero, L., *Biochem. Biophys. Acta* **86,** 412 (1964).
Stefanye, D., Schantz, E. J., Spero, L., *J. Bacteriol.* **94,** 277 (1967).
Sugiyama, H., DasGupta, G. R., Yang, K. W., *Proc. Soc. Exp. Biol. Med.* **143,** 589 (1973).
Uemura, T., Sakaguchi, G., *Jap. J. Vet. Sci.* **33,** 82 (1971).
Wagman, J. *Arch. Biochem.* **50,** 104 (1954).
Wagman, J., *Arch. Biochem. Biophys.* **100,** 414 (1963).
Wagman, J., Bateman, J. B., *Arch. Biochem.* **45,** 375 (1953).
Weil, L., Seibles, T. S., Spero, L., Schantz, E. J., *Arch. Biochem. Biophys.* **68,** 308 (1957).

Received for review April 10, 1973. Accepted October 4, 1973. Presented at the Symposium on Toxic Proteins, Peptides, and Related Substances, Division of Agricultural and Food Chemistry, 165th National Meeting of the American Chemical Society, Dallas, Texas, April 1973.

Allergens

Joseph R. Spies

The problem of isolation and characterization of allergens is complicated for many reasons, some of which are discussed. Included are: the present state of classification of allergens; the significance of the original elucidation of the polysaccharidic protein nature of the cottonseed and castor bean allergens in relation to isolation and chemistry of allergens in general; and the related concept that chemically different compounds may share common antigenic and allergenic determinants. Food allergy is especially complicated in that the products of digestion may trigger an allergic response. Based on demonstration of 12 new antigens generated by pepsin hydrolysis of milk proteins, we have suggested that the body immune system may be exposed to at least 100 new antigens, all of which are potential allergens, on ingestion of milk. Our results may explain why foods, in many cases, do not give a skin reaction on persons who give an immediate-type allergic response on ingestion of the food.

The objectives of this review are to discuss: problems involved in studying the chemistry of allergens; emerging concepts of the chemical nature of allergens and their determinants; clarification of the significance of work on oilseed allergens; and current research at Dairy Products Laboratory (DPL) on attempted elucidation of the mechanism of the allergic response to ingested milk proteins in particular and other foods in general.

The term "allergie" was used first by von Pirquet (1906) to denote an altered capacity of a human to react to a second injection of horse serum. Since that time "allergy" has generally been used to describe all forms of hypersensitivity in man. An allergen may be defined as "an ordinarily harmless substance present in the diet or environment, capable of producing such diseases as asthma, hay fever, eczema, and gastrointestinal upsets upon contact with a previously sensitized person." Allergy is the body response to an allergen antibody reaction which triggers the release of chemical mediators of hypersensitivity, namely histamine, serotonin, and acetylcholine, as well as larger compounds, SRS (slow reacting substance), and the plasma kinins (Austen, 1965). There are essentially three types of allergy. In atopic allergy, immediate-type symptoms appear in a few minutes up to 60 min after exposure. This type of reaction is initiated by the specific reaction of allergen with reagin [also called skin-sensitizing antibody, homocytotropic antibody or IgE (Ishizaka and Ishizaka, 1970)]. In delayed allergy, symptoms appear in from a few up to 96 hr after exposure. This reaction is initiated by specific reaction between allergen and small lymphocytes (Raffel, 1965). Anaphylactic-type allergy may occur in seconds to minutes after exposure with violent, sometimes fatal, symptoms. This reaction is initiated by specific reaction between allergen and IgG or IgE antibodies. This paper is mainly concerned with immediate-type allergy.

Reported overall incidence of allergy varies, but 10% of the general population is cited most often.

A comprehensive review of the chemistry of allergens is beyond the scope of this paper both because of space limitations and because recent publication of a monumental, critical review containing 558 references would make such an attempt redundant from a literature reference standpoint. Berrens (1971), in a monograph of 298 pages titled "The Chemistry of Atopic Allergens," has reviewed the subject of allergens in eight categories, namely the pollens, including timothy and cocksfoot (Augustin and Hayward, 1962), ryegrass (Johnson and Marsh, 1966a), alder (Herbertson *et al.*, 1958), ragweed, the greatest single cause of allergy from a natural source in the U. S. (Goldfarb, 1968; King *et al.*, 1967; Richter and Sehon, 1960; Robbins *et al.*, 1966; Underdown and Goodfriend, 1969); allergens from vegetable dusts and fibers, including ipecac (Berrens and Young, 1963), liquorice (Berrens, 1964), pyrethrum (Zucker, 1965), kapok (Berrens, 1966a; Coulson *et al.*, 1944), and cotton linters (Berrens and Versie, 1967; Coulson and Stevens, 1940); allergens from seeds, mainly cottonseed (Spies *et al.*, 1940b, 1960) and castor beans (Panzani and Layton, 1963; Spies, 1967; Spies and Bernton, 1962; Spies and Coulson, 1964); allergens from epithelial tissues such as horse dandruff (Stanworth, 1957), human dandruff (Berrens *et al.*, 1966), feathers (Berrens,

Dairy Products Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250.