ON THE TEST FOR NON-ANTIGENICITY OF PROTEIN HYDROLYSATE

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A modification to increase the sensitivity of the U.S.P. test for non-antigenicity of parenteral preparations of protein hydrolysate has been developed. The method lies in sensitizing guineapigs with doses of protein hydrolysate and challenging the sensitized animal with a sensitizing solution containing the homologous protein and prepared according to U.S.P. XV. Different batches of protein hydrolysate have been subjected to both the original U.S.P. and the modified test, and the results show that the latter method is more suitable to detect minute traces of antigen, which are likely to evade the usual test. It has further been shown that increasing the hydrolysis or treatment with a suitable ion-exchange resin leads to decontamination of an otherwise antigen-contaminated preparation of protein hydrolysate.

The injection of protein hydrolysate is a preparation which has established itself in modern therapeutics (Elman, 1945), in the control of malnutrition (Krishnan *et al.*, 1944), hypoproteinemia, and other associated conditions (Hodges, 1947 ; Koop *et al.*, 1947). In order to see that the solution, when injected is not likely to cause an anaphylactic reaction, a test for studying the non-antigenicity of protein hydrolysate is laid down in the United States Pharmacopoeia XV.

While applying the U.S.P. test for non-antigenicity to some preparations of meat protein hydrolysate which had shown a few clinical side reactions, certain observations were made in this laboratory which suggested that under different conditions of study, preparations which passed the usual U.S.P. test, could be shown to produce reactions similar to those produced by antigen-antibody combination in sensitised guineapigs (Bose *et al.*, 1959). As the findings were likely to have a bearing on the official test for non-antigenicity, a more detailed work was undertaken in this respect. The present paper is concerned with the results of these investigations.

METHODS

Materials.—Meat was taken as the material for the preparations of protein hydrolysate injection. Several batches were prepared by suitable enzymic (papain-trypsin) digestion of meat, the degree of hydrolysis varying generally

from 49 to 52 per cent. The solution was made reasonably free from native protein, as shown by the failure of any precipitation with 10 per cent trichloracetic acid. The material was adjusted with regard to pH and nitrogen, glucose and salt contents, filled in 25 ml ampoules, sterilized by heating in the autoclave at 10 lbs. for half an hour and preserved in the cold. A typical batch of protein hydrolysate was taken for resin-treatment.

Procedure.—Solution was prepared according to the method laid down in U.S.P. XV, using the same protein as that used in the preparation of protein hydrolysate. Different groups of healthy male guineapigs weighing between 400 and 500 g were sensitized by intraperitoneal injection of the test samples in doses varying from 2 to 6 ml. The injections were made on the 2nd, 4th, and 6th day of each of the two successive weeks, according to a schedule.

Each sensitized animal was challenged, between 30 and 37 days after the last sensitizing dose, with intravenous injection of the respective antigen into the dorsal vein of the penis.

Two different procedures were adopted for the study of non antigenicity. In procedure I, the animals were sensitized with injections of the sensitizing solution, and challenged with protein hydrolysate injection, according to the procedure of U.S.P. XV. In procedure II, which is a modified one, sensitization was caused by repeated injections of protein hydrolysate and the animals challenged with the sensitizing solution.

As intravenous injection of any protein breakdown product may give rise to some non-specific shock, control injections of both protein hydrolysate and the sensitizing solution were also made at the same time to allow comparison of reactions, if any, between the sensitized and non-sensitized animals.

Treatment of protein hydrolysate with ion-exchange resins.—Cation exchange resin was used to remove some amines and some basic protein bodies from the protein hydrolysate (Dutta and Bose 1958a, 1958b). On this principle the protein hydrolysate has been treated with cation-exchanger in the following way. A cation exchange resin (sulphonated phenolic type) was regenerated with 5 per cent hydrochloric acid (3 bed volume) followed by working with distilled water to remove the excess acid. Another cation exchanger Zeokarb 226 (Permutit and Co.), containing only carboxyl groups as the ion active groups was regenerated in two forms—one in H with 5 per cent hydrochloric acid (3 bed volume) and in other case as Na with 2 per cent sodium

hydroxide (3 bed volume) followed by distilled water to wash off the acid or the alkali. In the latter case the pH in the column was maintained between 7.0 and 8.0 Then aliquots of protein hydrolysate were percolated through these three columns, the percolates being marked as (i) NCl, (ii) Z-H and (iii) Z-Na respectively. These three treated protein hydrolysates underwent the tests for non-antigenicity.

Scoring for anaphylactic reaction.—The typical symptoms of anaphylactic reaction were (i) licking of the nose or rubbing of the nose with the fore-feet, (ii) ruffling of hairs, (iii) laboured or depressed breathing and (iv) sneezing or coughing. The reactions, if severe, led to death of the animal. The intensity of the reaction varied from animal to animal, and therefore, in order to get a more critical idea, a system of scoring was adopted in these experiments by putting 2 marks for each of the test symptoms. For death following typical symptoms, or for severity of the listed symptoms an extra mark of 2 was allotted. The total score thus came to 10 A minimum score of 4 was taken to denote development of typical anaphylaxis in the animal.

RESULTS

It can be seen from tables that the different batches of protein hydrolysate though passing the usual U. S. P. test (Table I), showed definite antigenic reactions when the animals were sensitized with the protein hydroly-

TABLE I

Test for non-antigenicity of meat protein hydrolysate according to U. S. P. XV. (Procedure I) Male guineapigs 300-350 g sensitized with sensitizing solution and challenged with protein hydrolysate

Protein hydrolysate	No. of animal	Dose in ml/number of sensitizing injection	Challenging solution, ml	Reaction score	Remark
Batch I	1	3/6	3.0	1	
	2	3/6	3.0	2	Complied with
	3	3/6	3.0	2	U. S. P.
	4	3/6	3.0	3	
Batch II	1	3/6	3.0	1	Complied with
	2	3/6	3.0	1	U. S. P.
	3	3/6	3.0	1	
Batch III	1	3/6	3.0	2	Complied with
	2	3/6	3.0	3	U. S. P.
	3	3/6	3.0	2	

sate concerned, and challenged with the antigen in the form of sensitizing solution (Table II). This finding clearly suggests that inspite of hydrolysis (49 to 52 per cent) the enzymic preparation possibly contains minute traces of protein which are enough to cause sensitization in animals.

TABLE II

Test for non-antigenicity, according to Procedure II. The samples were the same as those tested in Table I. Male guineapigs 400-500 g sensitised with protein hydrolysate and challenged with sensitizing solution

Protein hydrolysate	No. of animal	Dose in ml/number of sensitizing injection	Challenging solution, ml	Anaphylactic reaction	Reaction score (Max=10)	Remark
Batch I	1	3/6	3.0	Very severe (death)	10.0	Signified presence of antigen.
	2	3/6	3.0	Very severe (death)	10.0	
	3	3/6	1.5	Very severe	9.0	
	4	3/6	1.0	Death	10.0	
	5	3/6	0.5	Severe	8.0	
	6	3/6	0.5	Severe	8.0	
Batch II	1	3/6	3.0	Very severe	10.0	Signified presence of antigen.
	2	3/6	1.5	Very severe	10.0	
	3	3/6	1.0	Very severe	10.0	
	4	3/6	0.5	Severe	8.0	
Batch III	1	3/6	1.0	Severe	8.0	Antigen present but, quantity smaller than the other two batches.
	2	3/6	0.5	Moderate	6.0	
	3	3/6	0.5	Severe	8.0	

In order to see whether a higher hydrolysis of protein would result in freedom from such antigenic stimulation, another batch of protein hydrolysate was prepared with a higher degree of hydrolysis and subsequent purification. Table IV summarises the results of animal inoculations with these preparations. The animals were sensitized with 3 ml of the test materials, and challenged with varying doses of the sensitizing solution or the protein hydrolysate as the case might be.

TABLE III

Control experiments to determine the suitability of the antigenic solution

Test for antigenicity						Test for non-specific reaction (peptone shock after I.V. Inj, of test antigen	
Test antigen	No. of animals	Sensitized with	Challenged with (3 ml)	Anaphylactic reaction	Mean reaction score (Max=16)	No of animals	Reaction
Senstitizing solution	6	Sensitizing solution	Sensitizing solution	Very severe Death in 4	10.0	3	Nil
Protein hydrolysate	3	Protein hydrolysate Batch—I	Protein hydrolysate Batch—I	Insignificant	1.3	3	Nil
	3	Protein hydrolysate Batch—II	Protein hydrolysate Batch—II	Insignificant	2.0	3	Nil
	3	Protein hydrolysate Batch—III	Protein hydrolysate Batch—III	Insignificant	1.0	3	Nil
	3	,,	Egg albumin solution	Nil	0.0		
Egg albumin solution 5%	3	Sensitising solution	Egg albumin solution	Nil	0.0	3	Slight
	3	Egg albunim solution	Egg albumin solution	Very severe Death in 2	10.0		

It can thus be seen (Table IV) that the higher degree of hydrolysis has led to the loss of the sensitizing antigen, which usually is found to contaminate a preparation of lower hydrolysis (Table II).

Ion-exchange resins are frequently used to purify complex preparations. Considering that proteins could be adsorbed by treatment with some such resins, a batch of protein hydrolysate was treated with 3 types of resins, and the results of these preparations are summarised in Table V.

TABLE IV

Result of test for non-antigenicity with protein hydrolysate of higher degree of hydrolysis

No. of G. pigs	Sensitized with dose	Challenged with	Nature of reaction	Mean score (Max=10)	Remark
6	Sensitizing solution 2 ml	Sensitizing solution 1-2 ml	Severe with death	9.5	
6	Sensitizing solution 3 ml	Protein hydrolysate 1-2 ml	No reaction	0.5	Complied with U.S.P. test
3	Protein hydrolysate 3 ml	Protein hydrolysate 1-2 ml	No reaction	0	No sensitization with homologous material
6	Protein hydrolysate 3 ml	Sensitizing Solution 1-2 ml	Mild anaphylactic reaction in 2 others showed no reaction	2	Complied with modified test

TABLE V

Effect of treatment of enzymic protein hydrolysate with ion-exchange resins. Sensitization (Procedure II) caused by 6 tri-weekly injections of 2.5 ml intraperitoneally Assaulting done by intravenous injection of 2 ml of antigen challenging

Sensitizing solution	No. of animals	No. of injection	Challenging with	Anaphylactic reaction	Mean reaction score	Remark
Protein hydrolysate C18260	2	3	Sensitizing solution	Very severe with death	10.0	Did not comply with the modified test
	2	6	,,	,,	10.0	
NCL	2	3	,,	Very severe	8.0	Did not comply with the modified test
	2	6	,,	,,	8.0	
Z-H	2	3	,,	,,	7.0	Did not comply with the modified test
	2	6	,,	,,	8.0	
Z-Na	2	3	,,	Very slight	2.0	Passes the modified test
	2	6	,,		2.5	

DISCUSSION

It is significant to observe from the results of the experiments that, while antigen-sensitized animals failed to react when assaulted with homologous protein hydrolysate, animals sensitized with the latter showed typical and severe anaphylactic reactions when challenged with even a small dose of the antigen (0.5 ml). Protein hydrolysate-sensitized animals, however, did not present any significant anaphylactic reaction when challenged with the same material in doses upto 3 ml of the preparation. This failure of anaphylaxis in sensitized animals is possibly due to the low content of antigen in the assaulting dose of protein hydrolysate and which is insufficient to bring about the type reaction in the animals, whether sensitized with the type antigen in the form of the sensitizing solution, or with the homologous enzymic protein hydrolysate solution. It has been shown by an earlier worker (Topley, 1933) that it requires a relatively large dose of antigen to elicit an anaphylactic reaction in sensitized animals. The antigen-antibody reaction is commonly believed as a quantal phenomenon, and a definite relationship between the dose and effect in experimental anaphylaxis in guineapigs has been recently observed (Achari and Choudhuri, 1959). For sensitizing, however, a very small dose of antigen is often sufficient, though the amount of sensitization may vary with the type of the antigen as well as with the test animal, such as the guineapig, rabbit or dog.

The results of the present experiments also corroborate the above facts. It has been shown that a state of antigenic sensitization can be brought about by a protein hydrolysate containing a relatively low amount of protein, which by itself could not be detected in the U. S. P. test for non-antigenicity. The presence of a state of hypersensitiveness with the same preparation, however, can be definitely demonstrated if the challenging dose is administered in the form of the sensitizing solution which contains a much greater content of the homologous protein. From this, it appears reasonable to infer that there is a possibility for improving the U. S. P. test for non-antigenicity. It has been shown that the U. S. P. test when applied as a routine would frequently fail to detect the presence of antigen in an enzymic protein hydrolysate, with the result that some unwanted samples would pass, particularly if the challenging dose is given in the form of protein hydrolysate. Even if a large challenging dose of protein hydrolysate is found to give a typical reaction it may be difficult to distinguish it from non-specific peptone shock which can easily be brought about by a protein breakdown product in solution (Manwarung *et al.*, 1933). A control injection of protein hydrolysate in normal non-sensitised guineapigs simultaneously with the challenging

of the sensitized animals would put the U. S. P. test in jeopardy because of this complicating factor. On the other hand, if the test is performed in the reverse way and according to procedure II, laid down in Table II, by sensitizing the animals with protein hydrolysate and challenging them with the sensitizing solution, (antigen), it would be possible to detect even a minute trace of antigenicity in the preparation without any possibility of complications being brought about by non-specific shock and reaction.

This modified procedure will make the U. S. P. test much more sensitive to detect the presence of antigen. It would conform to all the characteristics necessary for an antigen-antibody reaction. That the procedure deals with a typical anaphylactic reaction is shown by the failure of other type antigens, such as egg albumin, to react with protein hydrolysate-sensitised animals (Table III), and the condition of specific desensitization brought about in the animals after the survival of the shock.

The only point against the injection of sensitizing solution for challenge lies in the fact that, if improperly made the solution may be contaminated with an appreciable amount of histamine, which is known to mimic typical anaphylactic reaction in animals (Dragstedt, 1945). Since amounts varying from 0.5 to 3 ml of the antigen solution show no typical anaphylactic reaction (Table III), it would be reasonable to assume that histamine shock, which forms one of the characteristic features of acute anaphylaxis (Dale, 1929), will not interfere with the test if the dose is kept within the suggested level.

Judging the different preparations of protein hydrolysate, on the basis on the modified test for non-antigenicity (Procedure II) it appears that antigenic contaminations from enzymic hydrolysate might he removed by either increasing the degree of hydrolysate to 65 per cent or above, (Table IV), or by treating with an ion-exchange resin, such as, Zeocarb (Na form) (Table V). The hydrogen form of these resins, however, appears unsuitable for such purification.

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