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THE CHEMISTRY OF ALLERGENS

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XXI. Eight new antigens generated by successive pepsin hydrolyses of bovine β -lactoglobulin

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β -lactoglobulin was hydrolyzed for 6 successive 8 minute periods during which approximately 90 per cent of the protein was split into fragments with a molecular weight of 12,000 or less. Six dialysates and 6 endofractions were separated and analyzed for the presence of antigens distinct from that of β -lactoglobulin with the use of the Schultz-Dale technique and gel diffusion analysis, respectively. All of the dialysates contained common nonprecipitating new antigens. The first dialysate did not contain all of the new antigens common to the other 5, indicating at least 2 new antigens in the dialysates. Six precipitating new antigens were demonstrated in the endofractions. Precipitation of 2 of these was inhibited by β -lactoglobulin; Four were not so inhibited. By analogy to β -lactoglobulin, if pepsin hydrolysis generated at least 8 new antigens from each of the 12 to 14 known milk antigens, the immune system of the body would be exposed to about 100 potential allergens upon ingestion of milk. These results may explain why milk and undoubtedly other foods, in many cases, do not cause skin reactions on persons who give an immediate allergic response on ingestion of the food. Such persons may be sensitive to these new antigens formed by pepsin in the stomach during digestion.

In this study the term "new antigen"* is defined as an antigen with a specificity distinct from that of the protein from which it was generated. In a previous study, Spies and associates¹ demonstrated a new antigen in the dialysate of the 8 minute pepsin hydrolysate of each of 4 milk proteins, namely, β -lactoglobulin, α -lactalbumin, bovine serum albumin, and casein. The endofraction of the dialyzed hydrolysate of bovine serum albumin contained a second, new antigen, but no new antigens were demonstrated in the corresponding endofractions of the other proteins.

The purpose of this study was to determine whether one or several new antigens are generated by simulated stomach digestion (6 successive 8 minute pepsin hydrolyses) of β -lactoglobulin. β -Lactoglobulin was chosen for this study as a milk protein possessing major allergenic activity.

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*It is recognized that the term "new antigen" may represent a mixture of more than one new antigen not distinguishable in these tests.

EXPERIMENTAL PROCEDURE

Materials*

Crystallized β -lactoglobulin (73 Gm.) from Pentex Corp. was purified by 2 additional recrystallizations from which 46 Gm. of β -lactoglobulin was obtained. The nitrogen content of the recrystallized β -lactoglobulin was 13.8 per cent, air-dried basis. This sample contained no α -lactalbumin, bovine serum albumin, or casein as determined by gel double-diffusion analysis with the use of rabbit antisera raised against α -lactalbumin, bovine serum albumin and casein. Pepsin was twice recrystallized swine pepsin obtained from Worthington Biochemical Corp. Dialysis tubing was a seamless, viscose, cellulose product, which, according to the supplier (Arthur H. Thomas), retained molecules with a molecular weight of 12,000 and higher.

Methods

Nitrogen. Nitrogen was determined by a Kjeldahl micromethod.

Pepsin hydrolyses. The basic design of these experiments consisted of hydrolysis of β -lactoglobulin with pepsin (2 per cent of the weight of each substrate) for 8 minutes at pH 2. The reaction was stopped, and the hydrolysate was separated into 2 fractions by dialysis. The dialysate and the endofraction were isolated by lyophilization. The endofraction from each hydrolysis was then similarly rehydrolyzed with pepsin, and 500 mg. of each endofraction was reserved for immunologic analysis. This procedure was done 6 successive times. Details of the first hydrolysis and dialysis follow as an example.

β -Lactoglobulin (42 Gm.) was suspended in 360 ml. of water at about 8° C. and dissolved by rapid stirring during addition of 120 ml. of cold 0.5N HCl. The solution was warmed to 37° C. and the pH adjusted to 2.0 by dropwise addition of 0.5N NaOH or HCl. To the stirred solution was added 42 ml. of a water solution containing 840 mg. of pepsin. The pH was maintained at 2.0 ± 0.05 by manual addition of 0.5N HCl for 8 minutes at $37^\circ \pm 1^\circ$ C. The solution was then poured onto ice cubes which lowered the temperature to about 9° C. in one minute. To the cold slurry was added with stirring the calculated amount of 0.5N NaOH to neutralize the added HCl. The temperature was then raised to 25° C. and the pH adjusted to 7.5. The hydrolysate solution was lyophilized. The hydrolysate then was dissolved in water and the solution dialyzed with toluene preservative against 4 approximately fivefold volumes of water changed at intervals of 2 to 3 days. The combined dialysates from each hydrolysis were lyophilized. Dialysates from successive hydrolyses were designated D1 to D6, respectively, as shown in Table I. The endosolution was lyophilized. Endofractions from successive hydrolyses were designated E1 to E6, respectively, as shown in Table I.

General Schultz-Dale technique. Virgin, female guinea pigs, weighing about 225 grams, were sensitized on the same day by subcutaneous injections (nuchal area) with two 0.5 ml. volumes of fraction emulsified with Freund's complete adjuvant. Dialysate fractions were dissolved in water and emulsified in a water-oil ratio of 1:1. The sensitizing dose of dialysate contained 2 mg. of dialysate nitrogen. The incubation period was at least 28 days. Challenge doses were administered in terms of total nitrogen in a 50 ml. Dale bath. The basic Schultz-Dale technique used, which utilized uterine horns of the sensitized guinea pigs, has been described by Coulson.² In this study, each of the 2 uterine horns of the sensitized guinea pig was cut into 2 equal parts called strips. The ovarian strips and the vaginal strips were used in pairs, one in each bath.

Rabbit antisera. Rabbits were immunized by injection of 0.25 ml. of the fraction solution emulsified with Freund's complete adjuvant in each of the 4 footpads. Endofractions were dissolved in physiologic salt solution and emulsified in a water-oil ratio of 1:1.4. The immunizing dose was 5 mg. of endosolid. After an incubation period of 28 days, a single 1 ml. booster dose was administered intravenously or intra-abdominally. The booster dose contained 5 mg. of endosolid. Rabbits were bled 7 days after administration of the booster dose.

Gel double-diffusion technique. The Ouchterlony³ technique was used. Test and agar solu-

*The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

TABLE I. Data on successive pepsin hydrolyses of β -lactoglobulin

Hydrolysis No.	Starting material (Gm.)	HCl per Gm. of material* (mEq.)	Dialysate	
			Symbol	Yield† (Gm.)
1	Lg (42.0)	0.045	D1¶	4.6
2	E1 (39.0)	0.44	D2	13.6
3	E2 (23.4)	0.33	D3	8.9
4	E3 (12.4)	0.17	D4	4.8
5	E4 (8.0)	0.15	D5	2.7
6	E5 (4.8)	0.13	D6	1.7

*To maintain pH at 2.0 during hydrolysis.

†From starting material, inclusive of sodium chloride formed.

‡Nitrogen content inclusive of pepsin, air-dried basis.

§Based on total nitrogen in starting sample.

|| β -Lactoglobulin.

¶This D1 was redialyzed yielding 3.5 Gm. of the sample used in this work which contained 3.6 per

TABLE II. Results of Schultz-Dale tests for new antigens in D1 to D6 with the use of guinea pigs which were sensitized with D2

Challenge fractions	No. animals tested	Results		
		No. animals showing NA* in both dialysate fractions	No. animals showing NA* in D2 after desensitization with other dialysate fractions	No. animals not used†
D3	3	3	0	
D2				
D4	2	2	0	
D2				
D5	4	2	0	2
D2				
D6	5	3	0	2
D2				
D1	6	5	5	1
D2				

*NA = new antigen.

†Strips too irritable or not sensitive to one or both D2 and other D fraction.

tions were made up in 0.9 per cent saline buffered at pH 7.5 and contained 0.01 per cent Merthiolate. A single filling of wells with antiserum and test solution was used. Results were read daily and photographed after an optimal interval.

Pepsin autodigest controls. Because of the possible presence of autodigested pepsin in the hydrolysates, pepsin autodigest dialysate and endofractions were prepared for use as controls in the Schultz-Dale and gel diffusion tests, respectively, as described below.

Pepsin (8 Gm.) was dissolved in 100 ml. of water (pH of solution, 4.0) and the pH adjusted to 7.5. To simulate the dialysis period, this solution was stored with toluene preservative at room temperature for 9 days. The pH was then 7.3. The solution was then dialyzed against three 500 ml. volumes of water for 2, 2, and 5 days. The combined dialysates was lyophilized, yielding 2.2 Gm. of pepsin dialysate fraction. The endosolution was filtered and lyophilized, yielding 5.7 Gm. of pepsin endofraction. This pepsin endofraction (5 Gm.) was autodigested for 8 minutes with fresh pepsin similarly to the hydrolysis of the β -lactoglobulin

Nitrogen		Endofraction			
% ‡	% of total§	Symbol	Yield (Gm.)	Nitrogen	
				% ‡	% of total§
4.07	3.2	E1	39.5	13.4	92.4
8.93	23.4	E2	23.9	13.2	60.4
8.90	25.6	E3	12.9	12.9	53.8
8.40	25.2	E4	8.5	13.3	70.6
8.01	20.4	E5	5.3	13.1	69.4
8.2	22.3	E6	3.0	12.7	59.6

cent nitrogen.

substrates. From this treatment was obtained 1.5 Gm. of a pepsin autodigest dialysate fraction (PEPD, nitrogen content, 8.9 per cent) and 3.4 Gm. of a pepsin autodigest endofraction (PEPE, nitrogen content, 12.5 per cent).

Test procedure for new antigens in dialysate fractions. Preliminary tests showed that D1 and D2 contained a common new antigen and that D2 was a better sensitizer than D1. The yield of D2 was the highest of the 6 dialysate fractions. Hence, D2 was selected as a reference standard to determine if D1 and D3 to D6 contained the same new antigens as D2 and/or new antigens distinct from that of D2. This was done both with guinea pigs uniformly sensitized to D2 and with guinea pigs individually sensitized with D1 and D3 to D6. Details of the procedures used are shown in the results section. Challenges of strips of at least 3 nonsensitized guinea pigs were all negative with 10 μ g of nitrogen of each of D1 to D6.

RESULTS

Table I contains data pertinent to the preparation and identification of the 6 successive pepsin hydrolyses and dialyses of β -lactoglobulin, D1 to D6, and E1 to E6.

Table II contains a summary of results of Schultz-Dale tests for new antigen(s) in fractions D1 to D6 with the use of guinea pigs uniformly sensitized with D2. Results are illustrated in Fig. 1 with the use of D4 as an example. Since D2 was used for sensitization, the positive responses to both D2 and D4 after desensitization with M (mixture containing equal amounts of nitrogen of β -lactoglobulin, pepsin, and autodigested pepsin dialysate [PEPD]) showed that D2 and D4 contain common new antigen(s). The fact that D2 produced no response in strips desensitized with D4 showed that D2 did not contain new antigen not present in D4. Results similar to those illustrated in Fig. 1 were obtained with D3 to D6 with from 2 to 5 guinea pigs tested with each fraction. No results were obtained which indicated a new antigen not common to D2 in any of these fractions. In several cases, D2 gave a positive response in strips desensitized with D1. Consequently, the test for a common new antigen in D1 and D2 was modified to eliminate the possibility that the response of D2 after desensitization to D1 was caused by an excess of M components in D2 compared to that in D1. These results are illustrated in Fig. 2. The fact that D1

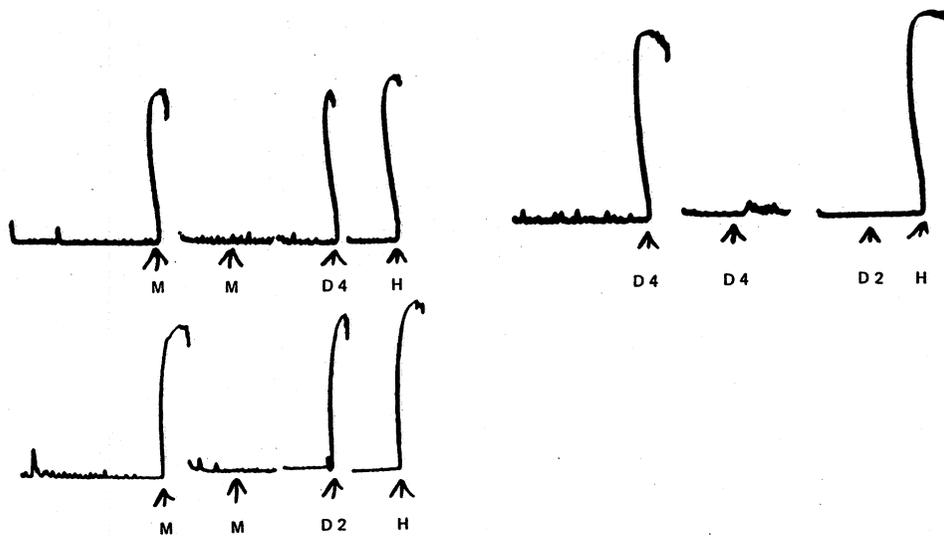


FIG. 1. Demonstration of common new antigens in D4 and D2 and the fact that D2 does not contain new antigens other than those present in D4 by Schultz-Dale technique. Sensitizing antigen, **D2**. Challenge doses in micrograms total nitrogen of test fractions: **M** (β -lactoglobulin, pepsin, and PEPD), each component, 10; **D3**, 10; **D2**, 10; **H**, histamine.

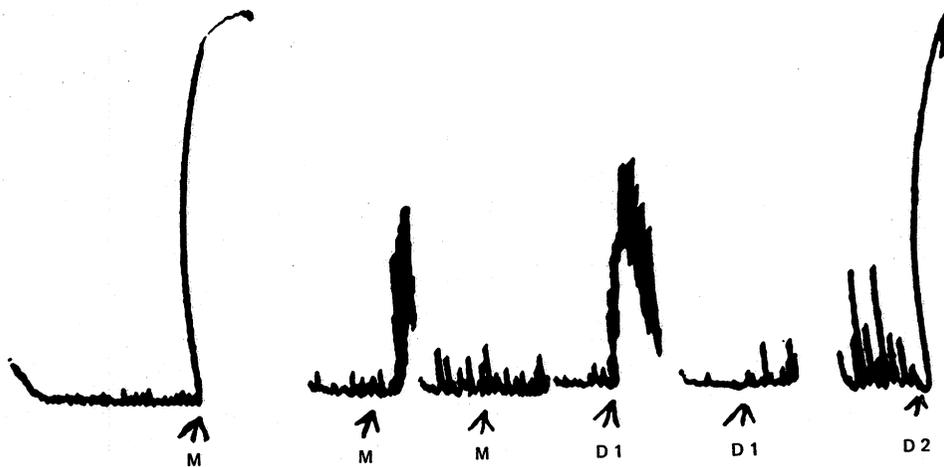


FIG. 2. Demonstration of common new antigen in D1 and D2 and the fact that D2 contains a new antigen other than that present in D1 by Schultz-Dale technique. Sensitizing antigen, **D2**. Challenge doses in micrograms total nitrogen of fractions: **M** (β -lactoglobulin, pepsin, and PEPD), each component, 10; **D1**, 10; **D2**, 10.

reacted positively after desensitization with M showed that D1 contained a new antigen common to D2; and that D2 reacted after desensitization with D1 showed that D2 contained additional new antigen not present in D1.

Table III contains a summary of results of Schultz-Dale tests for a new antigen in D1 to D5 with the use of strips from guinea pigs sensitized individually with D1 and D3 to D5 and uniformly challenged with D2. Results of

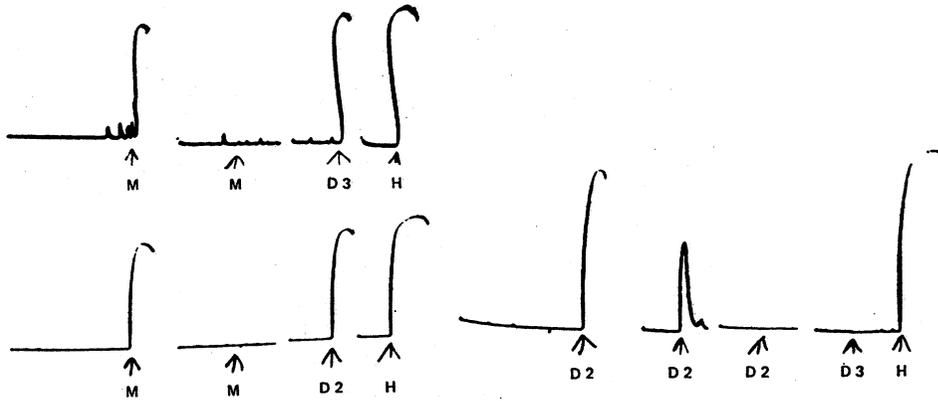


FIG. 3. Demonstration of common new antigens in D3 and D2 and the fact that D3 does not contain new antigens other than those present in D2 by Schultz-Dale technique. Sensitizing antigen, **D3**. Challenge doses in micrograms total nitrogen of fractions: **M**(β -lactoglobulin, pepsin, and PEPD), each component, 10; **D3**, 10; **D2**, 10; **H**, histamine.

TABLE III. Results of Schultz-Dale tests for new antigen in D1 to D5 with the use of guinea pigs which were sensitized with individual dialysate fractions

Fraction		No. animals tested	Results			
Sensitizing	Challenging		No. animals showing NA* in both dialysate fractions	No. animals showing NA* in dialysate fraction after desensitization with D2	No. doubtful	No. not used†
D1	D1 D2	2	2	0		
D3	D3 D2	4	2	0	1	1
D4	D4 D2	3	2	0		1
D5	D5 D2	2	2	0		

*NA = new antigen.

†Strips too irritable or not sensitive to one or both D2 and other dialysate fraction.

these tests are illustrated in Fig. 3 with the use of D3 as example. Since D3 was used for sensitization, the positive responses to both D2 and D3 after desensitization with M showed that D2 and D3 contained common new antigen(s). That D3 produced no response in strips desensitized with D2 showed that D3 did not contain new antigen(s) not present in D2. Results similar to those illustrated in Fig. 3 were obtained with D1 and D3 to D5 with from 2 to 4 guinea pigs each. There was an indication that D6 contained a new antigen in addition to the common new antigens present in D2 to D5 when D2 and D6 were tested by the procedure illustrated in Fig. 3. However, this possible new antigen was not counted as a third new antigen in the dialysates because of the inconclusive nature of the evidence.

Table IV shows results of tests for precipitating new antigens in E1 to E6 with the use of rabbit anti-E1 to E6 absorbed with β -lactoglobulin and PEPE

TABLE IV. Results of gel double-diffusion analysis of E1 to E6 for new antigen with absorbed E1 to E6 antisera and the effect of β -lactoglobulin inhibition on the precipitation

Antiserum*	Antigen†									
	Lg	P	PEPE	E1	E2	E3	E4	E5	E6	
NI‡	0	0	0	0	0	0	0	0	0	
Lg	1	-	-	1	1	1	1	1	1	
PEPE	0	1	2	1	1	2	2	2	2	
Abs. anti-E1	0	0	0	2	1	0	0	0	0	
Abs. anti-E1 (I)	-	-	-	1	0	0	0	0	0	
Abs. anti-E2	0	0	0	2	±	0	0	0	0	
Abs. anti-E2 (I)	-	-	-	0	0	0	0	0	0	
Abs. anti-E3	0	0	0	2	1-2	1	1	1	1	
Abs. anti-E3 (I)	-	-	-	0	0	0	0	0	0	
Abs. anti-E4	0	0	0	0	1-2	1-2	1-2	1	1-2	
Abs. anti-E4 (I)	-	-	-	0	1-2	1	1	1	1	
Abs. anti-E5	0	0	0	0	±	±	1	±	±	
Abs. anti-E5 (I)	-	-	-	0	0	0	0	0	±	
Abs. anti-E6	0	0	0	0	2	1	1	1	1	
Abs. anti-E6 (I)	-	-	-	0	2	1	1	1	1	

*Abs. = β -lactoglobulin-PEPE absorbed antiserum; (I), β -lactoglobulin-inhibited, absorbed antiserum.

†0, no precipitate; numeral shows number of lines or zones of precipitate; ±, inconclusive; -, not tested; Lg = β -lactoglobulin; P = pepsin.

‡Serum from nonimmunized rabbit.

TABLE V. Minimum number of precipitating new antigens in endofractions

Fraction	New antigens*	
	Lg-related	Lg-unrelated
E1	1	1
E2		2†
E3-E6	1	1

*Lg = β -lactoglobulin.

†From tests with Abs. anti-E6 and Lg-inhibited Abs. anti-E6.

(β -lactoglobulin-PEPE absorbed antiserum [Abs.] anti-E1 to E6) and Abs. anti-E1 to E6 inhibited with β -lactoglobulin. None of the antigens precipitated with nonimmunized rabbit serum. All endofractions precipitated with anti- β -lactoglobulin and with anti-PEPE, some giving 2 lines with anti-PEPE. Only anti-E6 precipitated with PEPE and pepsin showing that the pepsin and PEPE contents of E1 to E5 were not enough to immunize rabbits to these substances. Results of Table IV are summarized in Table V. New antigens in E1 to E6 related to the original specificity of β -lactoglobulin are precipitated with Abs. anti-E1 to E6 but not with Abs. anti-E1 to E6 when first inhibited with β -lactoglobulin. Those new antigens unrelated to the original specificity of β -lactoglobulin are precipitated with Abs. anti-E1 to E6 even when inhibited with β -lactoglobulin. An example of a β -lactoglobulin-related and a β -lactoglobulin-unrelated new antigen in E1 are shown in Figs. 4 and 5 in which the 2 lines of precipitate indicate 2 new antigens in E1. When the precipitin reaction was first inhibited with β -lactoglobulin, only the outer line of E1 precipitate remained (Fig. 5). In Fig. 5 it is apparent that E3 to E6 did not precipitate

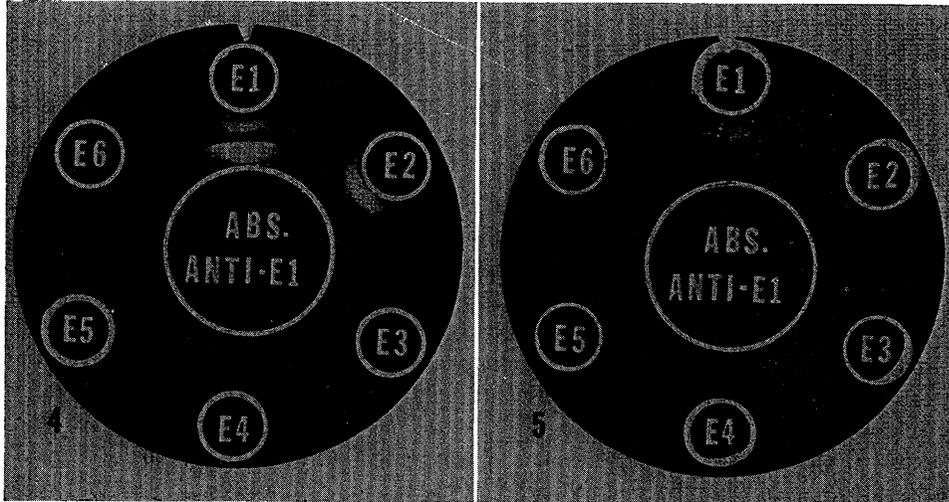


FIG. 4. Demonstration of precipitating new antigens in E1 and E2 by gel double-diffusion analysis. **Center well**, 0.5 ml. of Abs. anti-E1 (absorbed with β -lactoglobulin and PEPE); **peripheral wells**, 0.07 ml. of indicated endofraction, 0.5 mg. endofraction nitrogen per milliliter.

FIG. 5. Demonstration of β -lactoglobulin-related and -unrelated precipitating new antigens in E1 by gel double-diffusion analysis with β -lactoglobulin inhibition. **Center well**, 0.5 ml. of solution containing 2 mg. of β -lactoglobulin nitrogen per milliliter diffused for 24 hours. Solution in all wells then removed and replaced as follows: **center well**, 0.5 ml. of Abs. anti-E1 (absorbed with β -lactoglobulin and PEPE); **peripheral wells**, 0.07 ml. of indicated endofraction, 0.5 mg. of endofraction nitrogen per milliliter in each.

with Abs. anti-E1. Among the less-decisive precipitates obtained with E3 to E6 versus homologous Abs. antisera, E3 appeared to contain a β -lactoglobulin-related new antigen and E4 and E6 contained a β -lactoglobulin-unrelated new antigen. There may be additional new antigens in E3 to E6, but this determination will require further study.

An anomalous situation occurred in the testing of E2 for new antigens with Abs. anti-E3 to E6. As shown in Table IV, E2 contained little or no new antigen when tested with Abs. anti-E2. However, when E2 was tested with the use of both Abs. anti-E6 and Abs. anti-E6 inhibited with β -lactoglobulin, 2 lines of precipitate formed. E2 also gave more precipitate with Abs. anti-E1 and Abs. anti-E3 to E5 than with Abs. anti-E2. The explanation for this observation, which was checked independently by 2 observers, is not known.

DISCUSSION

A minimum of 8 new antigens has been demonstrated in the dialysis fractions of 6 successive 8 minute pepsin hydrolyses of β -lactoglobulin in a simulated stomach digestion. Two of the new antigens were common to dialysate fractions D2 to D6 which have a molecular weight of 12,000 or less. The dialysate fractions amounted to approximately 90 per cent of the starting β -lactoglobulin. Six of the new antigens occurred in endofractions E1 to E6 having a molecular weight of

over 12,000. The new antigens in fractions D1 to D6 were conclusively demonstrated by the Schultz-Dale technique. Fractions D1 to D6 did not produce precipitating antibodies in rabbits. The new antigens in fractions E1 to E6 were not very well demonstrable by the Schultz-Dale technique, but, in contrast to D1 to D6, fractions E1 to E6 produced precipitating antibodies in rabbits. By absorption and by β -lactoglobulin inhibition techniques it was demonstrated that 2 new antigens, one in E1 and one among the E3 to E6 fractions, retained some β -lactoglobulin-related antigenic determinants. Four new antigens, one in E1, two in E2, and one among the E3 to E6 fractions, contained antigenic determinants unrelated to β -lactoglobulin.

Hanson and Johansson⁴ and Hanson⁵ have reported that mature bovine milk contains 12 to 14 separate protein components which may lead to antibody production in man. If each of these antigens yielded 8 new antigens similarly to β -lactoglobulin, we can estimate that about 100 new antigens would be generated on ingestion of milk alone. If we project these findings on milk to all foods, it appears that the body's immune system may be exposed to a multitude of new antigens, all of which are potential allergens not present in the original foods, after ingestion of the food. Although we do not now know the sensitizing properties of these new antigens, it seems likely that some of them, at least, might act as allergic sensitizers in man in a manner similar to that of other low-molecular weight substances, such as drugs.

In the previous investigation¹ it was demonstrated that new antigen could be detected after only 1, 2, and 4 minutes of pepsin hydrolysis of total milk proteins. In this work it is apparent that common new antigens continue to be generated in the dialysate fractions (D1 to D6) over a period of at least 48 minutes during which 90 per cent of the original β -lactoglobulin was hydrolyzed. Since absorption of immunologically significant amounts of allergens is known to occur in a few minutes,⁶⁻⁹ this continuous production of common new antigens in the dialysates would tend to enhance the sensitizing potential of these new antigens.

Determination of the chemical and immunochemical nature of these new antigens would contribute to the elucidation of the mechanism of immediate-type allergic response to ingested proteins and possibly be the key to explain why many clinically food-sensitive persons do not exhibit a skin reaction to the undigested food proteins. They may be sensitive to one or more of the new antigens similar to those generated by pepsin hydrolysis as described here. Individuals who are skin reactive to the original proteins could also be skin reactive to these new antigens.

Further study of these new antigens from milk products is in progress.

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