

# IMMUNOLOGICAL STUDIES WITH PLASMA EXPANDERS DERIVED FROM HUMAN PLASMA \*

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During the course of immunological testing of several proposed plasma expanders derived from human plasma, it was observed that the processing of the expanders created a new protein which was antigenic in humans. All the procedures for processing the human plasma incorporate heat treatment for 10 hours at 60° C in the presence of suitable stabilizers. This step is included to inactivate the virus of hepatitis (1-4).

The reprocessed, heat-treated plasma preparations fall into three classes: 1) heated "whole" plasma from which little fibrinogen has been removed; 2) partially fractionated materials from which gamma globulin, fibrinogen and some lipoproteins have been removed; 3) albumin solutions. The stabilizers employed during the heating procedures were Na caprylate, Na acetyl tryptophanate, or dextrose.

In many cases these plasma expanders show evidence of major changes in their electrophoretic and ultracentrifugal patterns. In addition, some systemic reactions to infusions of the expanders have been noted (5). Because high molecular weight aggregates and possibly some denatured albumin-globulin complexes are formed during the preparation of these expanders, studies were undertaken to learn whether or not they are antigenic in humans.

The present report deals with the techniques employed for studying the antigenicity of the ex-

panders and with the methods for detecting the new protein antigen.

## MATERIALS AND METHODS

The materials that were studied for antigenicity in both rabbits and volunteers are summarized in Table I. The physicochemical data were furnished either by the manufacturers or by the Division of Biological Standards of the National Institutes of Health. Published procedures are available describing the preparation of SPPS<sup>1</sup> (6); the Mulford, Mealey and Welton material (7, 8); the Hoch and Chanutin preparation (9); the Cutter plasmanate (HTPF) (10); PPL (11); and PPF (12).

1. *Immunization of rabbits.* Rabbit antisera were obtained against the following preparations: Hyland heated plasma 230C-30; Mulford FFP9B; SPPS BD-21; Cutter heated plasma; USP heated plasma; and Chanutin albumin. The methods of immunization, dosages used and times of bleedings are summarized in Table II. All the preparations were tested after immunization of rabbits by the adjuvant technique. The adjuvant mixture was made by mixing an equal volume of plasma expander (diluted to about 5 per cent protein solution) with an equal volume of a mixture of Bayol F (9 parts) and Arlacel C (1 part) (13, 14). Dead mycobacteria (1 mg per ml) were also included. For immunization with alum-precipitated materials (SPPS and Mulford), the procedure of 4 injections per week for 4 weeks per course was followed, using a suspension of 1.5 mg protein per ml as described in Kabat and Mayer (15). All antisera were handled with sterile precautions and kept frozen until studied.

2. *Analysis of antisera.* As the main purpose of this phase of the study was to try to identify any "new antigens" produced during processing of human plasma, the Ouchterlony technique of agar diffusion as modified by Björklund was employed (16). Initially, the antisera were studied by placing the appropriate antiserum in the center well and varying dilutions of antigen in the outer wells. This arrangement was used to characterize the number and complexity of the antigen-antibody systems. The modification of Björklund employs,

<sup>1</sup> The abbreviations used are: SPPS—stabilized purified protein solution; HTPF—heat-treated protein fraction; PPL—pasteurized plasma protein-Lösung; PPF—plasma protein fraction.

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instead of agar, agar mixed with normal human serum to a final concentration of 25 per cent by volume. When antiserum against normal human serum proteins is placed in the center well, free diffusion of the many antibodies is prevented by the tremendous excess of antigen around the center well. Only antibodies that are not formed against normal human serum proteins can diffuse freely. In the presence of the appropriate concentration of "new antigen," a band or bands will appear, depending upon the number of precipitating systems present.

Each antiserum was studied by this technique. The only system found to be positive was that of the Chanutin albumin-antiChanutin albumin serum.

Additional agar diffusion studies (in the presence of normal human serum) were made with Chanutin albumin antisera in the center well and dilutions of all the other plasma preparations listed in Table I in the outer wells. Other materials studied with this antiserum were Hyland HSA (4 preparations) and the following sub-fractions of serum: Fr. II, III, IV-1, IV-4, IV-5, IV-8, IV-9, V, VI, ceruloplasmin and  $\beta_1$  metal combining globulin. Other studies employed serum-agar plates which were made with Hyland and USP heated and unheated plasmas.

The antiChanutin serum was calibrated by the quantitative immunochemical procedures developed by Heidelberger and co-workers (15). The calibrated antiserum was used to compare several serum albumins with the Chanutin preparation and also to determine the appropriate concentration of albumin to use for absorbing the antialbumin antibodies. The antiserum that was absorbed at equivalence with HSA was studied again in agar diffusion in the presence of normal human serum and the various human plasma preparations and also by the quantitative precipitin technique with Chanutin albumin. Two other antisera produced against placental albumin and Hyland Fr. V were included in the study. These were investigated in the manner described above for the antiChanutin serum.

3. *Electrophoretic studies.* a) *Continuous filter paper electrophoresis*<sup>2</sup> (17, 18). The following samples were fractionated on a Spinco hanging paper electrophoretic apparatus kept in a cold room: Chanutin albumin, Hy-

<sup>2</sup> The authors wish to thank Dr. William Merchant and Mr. Thomas Giglotti of the Department of Research Medicine, VA Hospital, Pittsburgh, Pa., for carrying out these separations.

TABLE I  
Some properties of heated and unheated human plasma preparations

Type of material	Preparation	Protein conc.	Stabilizer added	Electrophoretic data						Sedimentation data* (component)		
				Alb.	Fib.	$\alpha_1$	$\alpha_2$	$\beta_1$	$\gamma$	I	II	III
Whole plasma	Hyland Labs 230C30	%										
	Unheated			65								
	Heated††	5.2	5% dextrose	44	←12→			11	12	17.4(2.4),	6.7(16.6),	4.0(81)
					←47→			3	6			
Partially fractionated plasma	USP P52-0003			55					17			
	Unheated	4.7	5% fructose	35	←28→				10	27.5(4)	5.7(4)	4.3(86)
	Heated††				←55→							
	SPPS BD-21			65.2		6.2	10.0	14.1	4.5			
	Unheated	4.5	0.008 M -caprylate	65.6		4.2	25.2	4.6	1.8			
	Heated††											
	Mulford FFP9B††	3.6	0.008 M Na acetyl tryptophanate	81			←19→				4.0(100)	
	Cutter plasmanate (HTPF) (1242)	4.9	0.004 M acetyl tryptophanate	88.5		←7.8→		3.7			4.0(100)	
			0.004 Na caprylate									
	Swiss Red Cross (PPL)§ T45		0.004 M Na caprylate +5% glucose	66-70			30-34				4.0(98)	
	Auerswald Austrian-prepn. PPF (211356)	3.9		88	←7→			5	0			
Albumins	Chanutin 1309†	24	0.02 M acetyl tryptophanate	100							3 components	
			+									
	1334	25	0.02 M Na caprylate	100								
	Hyland HSA 490B4	25	Same as above	100								

\* Values in parentheses refer to per cent of respective component present in mixture.

† Preparation studied for antigenicity in volunteers.

†† Preparation studied for antigenicity in rabbits.

§ Prepared by desalting of plasma (ion exchange treatment).

TABLE II  
*Procedures used in immunization of rabbits*

Prepn.	Method of immunization	Rabbits	Dosage employed	Dates of bleedings
		no.	mg.	
Hyland 230C-30	Complete adjuvants*	3	26, 26† 26‡	Weekly, 6 wks after 2nd inj. Weekly, 5 wks after 3rd inj.
USP	Complete adjuvants	4	18,† 18,‡	Weekly, 5 wks after 2nd inj. Weekly, 5 wks after 3rd inj.
SPPS	Alum ppt—2 courses	4	50 mg/course	Wk after last inj. of each course
	Complete adjuvant	4	20, 20†	Weekly for 6 wks after 2nd inj.
Mulford	Alum ppt—2 courses	4	50 mg/course	Wk after last inj. of each course
	Complete adjuvants	4	20, 20† 20‡	Weekly, 4 wks after 2nd inj. Weekly, 4 wks after 3rd inj.
Cutter plasmanate (HTPF)	Complete adjuvants	5	25, 25† 25‡	Weekly, 3 wks Weekly, 4 wks
Chanutin	Complete adjuvants	4	25	Weekly, 5 wks

\* Complete adjuvants refer to mixture containing dead mycobacteria.

† Interval of 2 weeks between 1st and 2nd injection.

‡ Third injection given after last bleeding of previous course.

land heated and unheated plasmas. The samples were dialyzed overnight in the cold against veronal buffer (pH 8.6;  $\mu = 0.02$ ) before application to the curtain. The entire apparatus was stabilized overnight by application of buffer to the curtain (S & S paper no. 470). Fractionation was performed at 50 ma and 720 v. The feeding rate was adjusted to handle 15 ml of protein solution per 18 hours. The circulating buffer was further refrigerated by cycling through a cold bath. The temperature of the curtain never exceeded 18° C. The material which was collected in 32 tubes was diluted with about 5 to 10 ml buffer; 0.5 ml aliquots were heated for 20 minutes in a boiling water bath with 0.5 ml of ninhydrin. Following cooling, 4 ml of 50 per cent propanol was added, and the color that developed was read at 540 m $\mu$  and plotted. The various fractions were then lyophilized and reconstituted for agar diffusion studies.

b) *Immunoelectrophoresis*. Immunoelectrophoretic studies as described by Grabar and Williams (19, 20) were performed with antiChanutin albumin serum unabsorbed and absorbed with crystalline HSA and with normal human serum, respectively. An Arthur H. Thomas paper electrophoretic assembly was adapted to immunoelectrophoresis by employing glass plates and a 3 to 4 mm layer of Noble agar made with veronal buffer (pH 8.3;  $\mu = 0.05$ ). The plates contained 1) two circular wells in which Chanutin albumin was placed and then subjected to electrophoresis; 2) three rectangular wells to which the appropriate antiserum was added for development of the antigen-antibody bands. Diffusion of antiserum and antigen was allowed to proceed for several days in a cold room in a sealed system to prevent excessive loss of water and drying of the agar plates.

4. *Immunization of volunteers*. Five of the preparations were studied in man as follows: 50 ml of blood was obtained from healthy medical students before skin test-

ing with the appropriate material, including saline as a control. Skin reactions were read 15 minutes later. Twelve volunteers divided into 3 groups of 4 were tested with each material. Each group of 4 volunteers then received an intramuscular injection of 5, 10 or 20 mg of protein per injection 5 times over a 7 to 10 day period. Bleeding and skin testing of students were repeated approximately 10 days and 3 to 4 weeks after the last injection. Reactions of the students were reported and recorded appropriately.<sup>3</sup> Some of the volunteers were retested 2 months after the last injection; 500 ml bleedings from several of the subjects who showed some reactions were also obtained for further study. All serum samples were handled with sterile precautions and, in addition, "Merthiolate" (0.01 per cent) and phenol (0.25 per cent) were added.

5. *Analysis of human sera*. All the sera obtained were analyzed for precipitins, both by the quantitative micro precipitin technique of Heidelberger and MacPherson (21, 22) and by various agar diffusion procedures.

a) *Micro precipitin technique*. Before analysis, the sera were centrifuged overnight in the cold as described by Maurer (23). Three ml portions of pre- and post-immunization sera were measured into 8 ml conical centrifuge tubes calibrated at the 2.5 ml mark. Concentrations of the appropriate preparation containing from 3  $\mu$ g N to 700  $\mu$ g N were added to the sera. The tubes were mixed, capped, placed in a water bath at 37° C for 1 hour and then in the refrigerator for 10 to 14 days, the contents of the tubes being mixed daily. The tubes were then centrifuged and the precipitates washed and analyzed as described previously. Additional antigen

<sup>3</sup> A more detailed study is available in the files of the National Research Council, Committee on Plasma. A complete summary of the reactions will be furnished upon request.

was added to the supernatants and treated as above. Appropriate controls of serum plus saline and of antigen alone were included.

b) *Agar diffusion studies.* The sera were analyzed by the Oudin (24), Preer (25), and Ouchterlony (26, 27) techniques of agar diffusion as follows. The concentrations of overlaying "antigen" employed in the Oudin procedure were 200  $\mu$ g N, 500  $\mu$ g N and 1 mg N per ml. With the Preer technique, undiluted serum (0.02 ml) was overlaid with agar (0.6 per cent), which was in turn overlaid with undiluted "antigen" or with a 1:2, 1:8, 1:32, 1:128, or 1:512 dilution of the "antigen." When the Ouchterlony technique was employed, it was necessary to prepare the agar plates with glycine and veronal buffer as described by Halbert, Swick and Sonn (28). This procedure was employed to eliminate the haze that forms around undiluted human serum in the presence of buffered agar alone. Undiluted sera were studied in the presence of undiluted "antigen" or of a 1:2, 1:8, 1:32, 1:128, or 1:512 dilution of "antigen." Observations were made daily for at least 2 weeks.

## RESULTS

1. *Rabbit antisera.* The only antiserum which, in agar diffusion studies, indicated the presence in the plasma expanders of a new component not present in normal human serum was the Chanutin albumin rabbit antiserum. Dilutions of Chanutin albumin reacting with antiChanutin albumin serum produced a definite antigen-antibody band in the presence of serum-agar mixtures (Figure 1). Increasing the concentration of human serum in agar up to 50 per cent did not remove the band. This same band persisted even when the anti-albumin had been removed from the antiserum by

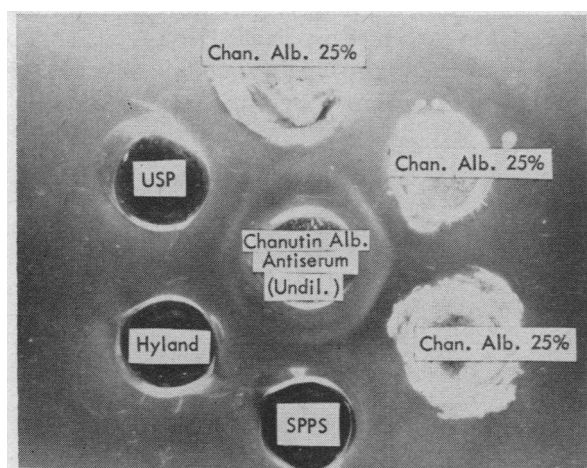


FIG. 1. AGAR DIFFUSION PATTERNS OF REACTIONS BETWEEN PLASMA EXPANDERS AND RABBIT ANTICHANUTIN ALBUMIN SERUM.

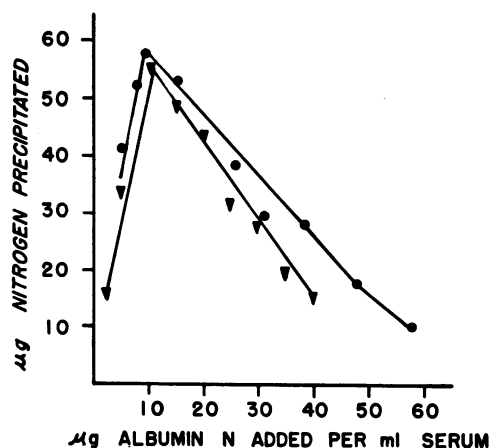


FIG. 2. PRECIPITIN CURVES OF CHANUTIN ALBUMIN ANTISERA (1:8 DILUTION) WITH CHANUTIN ALBUMIN (●) AND CRYSTALLINE HSA (▼).

absorption. Although none of the other plasma preparations showed such a reaction with its respective antiserum, many of them reacted with the antiChanutin serum. The SPPS, USP, Mulford and Hyland heated plasma preparations all gave joining bands with the Chanutin preparation (Figure 1) but did not react with their homologous antisera even when the samples had been concentrated threefold. The unheated USP and Hyland 230C-30 did not give bands. The heated and unheated Cutter preparations, Auerswald and normal Fr. V albumins from various sources did not give a positive reaction with the Chanutin antiserum. Serum agar plates made with heated Hyland or USP plasma inhibited the formation of the band, whereas the plates made with the unheated preparations did not.

The quantitative immunochemical study of the reaction of the Chanutin albumin with calibrated anti-HSA serum and the reaction of various albumins with antiChanutin sera (Figure 2) revealed no significant immunochemical differences. The antiserum against the Chanutin albumin preparation contained antibodies directed against many other proteins. When studied by agar diffusion (in the absence of normal human serum), positive reactions were obtained between the Chanutin antiserum and the following subfractions of serum: Fr. II, III, IV-1, IV-4, IV-5, IV-8, IV-9, VI,  $\beta_1$  metal binding globulin and ceruloplasmin.

The data presented in Figure 3 indicate that the Chanutin albumin antiserum does contain anti-

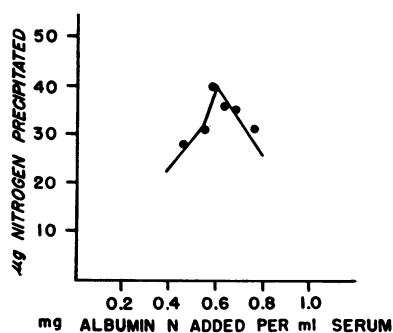


FIG. 3. PRECIPITIN CURVE OF CHANUTIN ALBUMIN ANTISERA ABSORBED AT EQUIVALENCE WITH CRYSTALLINE HSA WITH CHANUTIN ALBUMIN.

bodies precipitable by Chanutin albumin even after absorption at equivalence with crystalline HSA. This finding is further evidence of the heterogeneity of the Chanutin albumin preparation. If we assume an approximate ratio at equivalence of (antibody N)/(antigen N) of 4.5, it can be estimated that the Chanutin albumin contains at least 1 per cent of other proteins.

When the various fractions obtained by continuous paper electrophoresis from the Chanutin, Hyland and USP heated and unheated plasmas were allowed to react with antiChanutin serum in the presence of normal human serum and agar, the Chanutin and heated plasmas of USP and Hyland contained the new antigen. The antigen was located only in the fastest moving fractions of the albumin. Bands in agar diffusion were obtained with Hyland fractions 27, 28, USP fractions 29, 30 and Chanutin albumin fractions 28 to 30. Figure 4 shows the type of separations achieved and where the new antigen was located. The results obtained above were also

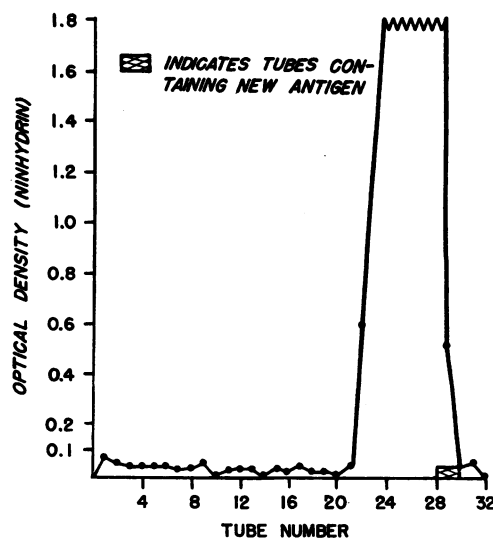


FIG. 4. CONTINUOUS PAPER ELECTROPHORETIC SEPARATION OF CHANUTIN ALBUMIN, 5 PER CENT (BARBITAL BUFFER PH 8.6,  $\mu = 0.02$ , 50 MA, 720 V).

confirmed by immunoelectrophoretic analysis. The antigen involved in the reaction with the absorbed antiChanutin serum was shown to be at the leading edge of the albumin area.

2. *Skin testing of volunteers.* With all the preparations studied, some cutaneous reactions were observed at the time of testing. There was only one marked systemic reaction following the skin testing. Skin reactions were considered positive when the wheal and erythema measured 10 mm or greater. In one individual, a marked delayed reaction was evident 24 hours after skin testing. A summary of the number of reactions with each material is presented in Table III.

3. *Immunochemical analysis of human sera.* Of all the sera analyzed by quantitative immuno-

TABLE III  
Summary of reactions in volunteers injected with plasma preparations

Material injected	No. of volunteers	No. of positive skin reactions			Systemic reactions
		Pre-imm.	10 Days after last inj.	3 Wks after last inj.	
SPPS BD-21	11	0	4	2	1 (Hives, dry and irritated throat)
Hyland 230-C-30	11	1	1	2	1 (Leg pains during immunization)
Mulford FFP9B	12	2	0	2	0
Cutter plasmanate	12	2	5	5	0
USP heated plasma	12	0	5	5	0

chemical techniques, only serum from one individual in the SPPS group gave a definite precipitate with the antigen. After removal of complement with a specific precipitate, a precipitate of 2.1  $\mu\text{g}$  N per ml was obtained. This value, which is at the limit of significance of the colorimetric method (29), includes not only "antibody" but also "antigen." What fraction of the total SPPS added is precipitated as "antigen" is not known. A subsequent bleeding obtained three weeks later showed a decrease in total N precipitated.

None of the agar diffusion techniques employed indicated the presence of antibody when the human sera were tested. This result was to be expected, as the level of antibody detectable by these techniques is usually of the order of several micrograms of antibody N (30).

#### DISCUSSION

The effect of heat treatment on the electrophoretic and immunological properties of serum and plasma has been known for some time (31-34). The extent of alteration in the physicochemical properties appears to be related to the complexing that occurs between molecules of albumin and of gamma globulin. In addition, increased heating leads to denaturation and to changes in the specificity of the serum proteins (35). The addition of appropriate stabilizers can reduce considerably the extent to which these reactions occur.

In the studies discussed here, an antigen not present in unheated plasma has been demonstrated in heated plasma. Although antibodies against the new antigen were produced in rabbits only following immunization with the Chanutin albumin, this antiserum could detect the antigen in many other heat-treated preparations of plasma.

The nature of this new antigen is of some interest. Originally it was thought that the new antigen would be associated with albumin-globulin complexes which were produced during the heating process. However, electrophoretic fractionation and immunoelectrophoresis indicated that the antigen was associated with the fast-moving fractions of albumin. The new antigen was definitely produced by heating (10 hours, 60° C) since it was not present in the unheated preparations. The joining of antigen-antibody bands indicated the similarity of this antigen in

all the preparations. Some preparations which were exposed to the same heating procedure did not exhibit this new antigen, i.e., Cutter plasmate (HTPF), Auerswald PPF, and several albumin preparations. This fact indicated that heating alone did not produce the new antigen. Although this "antigen" moves as a fast albumin, it is sufficiently different from albumin so that antibodies produced against it are not removed by normal human serum albumin (no cross reaction). The new antigen may be an albumin which has been partially hydrolyzed during heating. It was not possible to separate the new component in a Spinco ultracentrifuge (Preparative Model L) when run at 35,000 rpm for three hours. This observation indicates that the new antigen is not exceptionally heavy. Also, in the agar diffusion studies, the bands appeared close to the antibody well, possibly indicating that the material had a high diffusion constant.

One can only speculate about the interpretation of the data obtained with the immunization of volunteers. With one exception, no precipitating antibody was detectable in the human sera from the volunteers injected with the heated preparations. Because the nature of the response evoked by the injection of the solution of protein was frequently of a character making it difficult to distinguish between an immediate irritative response and one of hypersensitivity, a complete interpretation of the cutaneous reactions is not possible. However, cutaneous reactions such as itching at the skin test site during the course of immunization or at the ten day or three week testing dates, urticaria, a hot and irritated throat, and the "delayed reaction" of one subject suggest that some immunological phenomena may be responsible for the symptoms observed. The nature of the "antigen" responsible for the cutaneous and systemic reactions is completely unknown. It is not known whether the new antigen detected in many of these preparations with the rabbit antiserum (Chanutin albumin) is also the "antigen" responsible for the reactions in humans. It may well be that the artificially produced albumin-globulin complexes present in some preparations, or traces of denatured proteins in others, may be the antigen. That some individuals may react to normal human serum proteins that are different from their own is also plausible. Simi-

lar situations do exist in rabbits (36-38). Until we know more about the interpretation of the cutaneous reactions of individuals to highly purified substances, it is difficult to assess completely or adequately the reaction to materials consisting of a mixture of antigens. One can only speculate, therefore, about reasons for the cutaneous reaction to the materials employed. 1) Perhaps we are dealing with a classical "reagenic" type of antibody which gives positive skin reactions. The "antibody" may be present in such low concentrations that precipitation cannot be obtained. 2) The other possibility is that, in some instances, we may be dealing with "delayed hypersensitivity" to altered serum proteins.

#### SUMMARY

It has been demonstrated that many heated preparations of plasma contain a new antigen not present in the unheated materials. The usefulness of agar diffusion techniques in detecting this new component was shown. Immunization of volunteers with the human plasma preparations in several instances led to the appearance of reactions. The difficulty in attributing the reactions to any one antigen, as well as in interpreting the significance of the reactions, has been discussed.

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