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Effect of 2-Mercaptoethanol on Rabbit Pancreas Isoantibodies.* (31716)

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Previous reports(1,2) have described the production of isoantibodies to rabbit pancreas. These antibodies, which were produced by rabbits following intradermal injections of a simple saline extract of rabbit pancreas incorporated into complete Freund adjuvant, have been found to be both species- and organ-specific. They reacted with some but not all of a random selection of rabbit pancreas extracts, but never reacted with the pancreas extract of the antibody-producing rabbit itself. Later, the occasional appearance of autoantibodies to pancreas was noted in some rabbits receiving trichloroacetic acid precipitated pancreas extract in Freund adjuvant(3). No reaction was seen when normal rabbit serum was tested with pancreas extract.

Kidd and Friedwald(4) have described a naturally-occurring, heat-labile antibody that is present in normal rabbit sera and which reacts with extracts of many organs of normal rabbits (including the rabbit's own organs). Moreover, following immunization with pancreas extract, an increase in the titer of the reaction with autologous organ extracts such as gastrointestinal mucosa was observed(2). The present report describes some unusual immunochemical properties of these natural and induced rabbit antibodies to rabbit organ extracts.

Materials and methods. Preparation of antisera. Antisera were obtained by injecting rabbits with a saline extract of pooled rabbit pancreas extract which had been incorporated into an equal volume of complete Freund adjuvant. A total of 1 ml of the emulsion was injected intradermally at 3 week intervals, alternating between the 4 foot pads and the back of the neck. Trial bleedings from the central artery of the ear were taken just prior to each immunization. The final bleeding was taken from the heart. Antiserum to bovine serum albumin (BSA) was prepared following the same injection schedule.

Sucrose density gradient ultracentrifugation. Starting with 40% sucrose in phosphate buffered saline (pH 7.2) and following with 30%, 20%, and 10% solutions, 1.1 ml samples of each solution were layered into a 5 ml cellulose centrifuge tube. After equilibration for 2 hours at 4°C, 0.5 ml of serum in 10% sucrose solution was added. A few drops of brom phenol blue were added to the sucrose-serum mixture as a marker of albumin. After ultracentrifugation at 125,000 *g* for 18 hours in the Spinco model L ultracentrifuge using the SW-39 swinging bucket rotor, a hole was punched in the bottom of the centrifuge tube with a #25 gauge needle and the fractions were collected into tubes; 7 drops were allowed in each fraction.

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Mercaptoethanol treatment. The procedure for reduction and alkylation is that described by Deutsch(5). Equal volumes of 1% protein solution and 0.2 M 2-mercaptoethanol were mixed and allowed to stand at room temperature for 48 hours. The samples were then dialyzed against 0.1 M iodoacetamide at pH 8.0 for 72 hours at 4°C and against phosphate buffered saline pH 7.2 for 24 hours.

Serological tests. The methods of complement fixation, passive cutaneous anaphylaxis (PCA), and immunoelectrophoresis, as carried out in this laboratory have been described previously(1,2).

Experimental results. Nine anti-rabbit pancreas rabbit sera were subjected to sucrose density gradient ultracentrifugation at 125,000 *g* for 18 hours, and the fractions tested with rabbit pancreas extract by complement fixation. Controls of a known 19S antibody (anti-sheep red blood cell rabbit hemolysin) and a known 7S antibody (anti-BSA rabbit serum) were centrifuged simultaneously and tested with their appropriate antigens. The results of this procedure are shown in Fig. 1, where fraction 1 is taken from the bottom of the ultracentrifugation tube.

Each of the fractions of rabbit pancreas antiserum was tested for the presence of antibody to rabbit pancreas and to rabbit ileum extracts by complement fixation. In most instances, the largest amount of antibody was located in the fractions just preceding those fractions containing serum albumin as indicated by the presence of brom phenol blue. Anti-BSA was localized in the same area. On this basis, the sedimentation constant was judged to be approximately 7S. The sample of serum taken only 3 weeks after the first injection showed 2 antibody peaks; the more rapidly sedimenting population of antibody molecules corresponded to rabbit hemolysin, and probably represented a 19S component.

Following reduction with mercaptoethanol and alkylation with iodoacetamide, the complement-fixing ability of the rabbit pancreas antisera was assessed, and PCA and precipitating abilities examined. Experiments were carried out using both whole serum and the

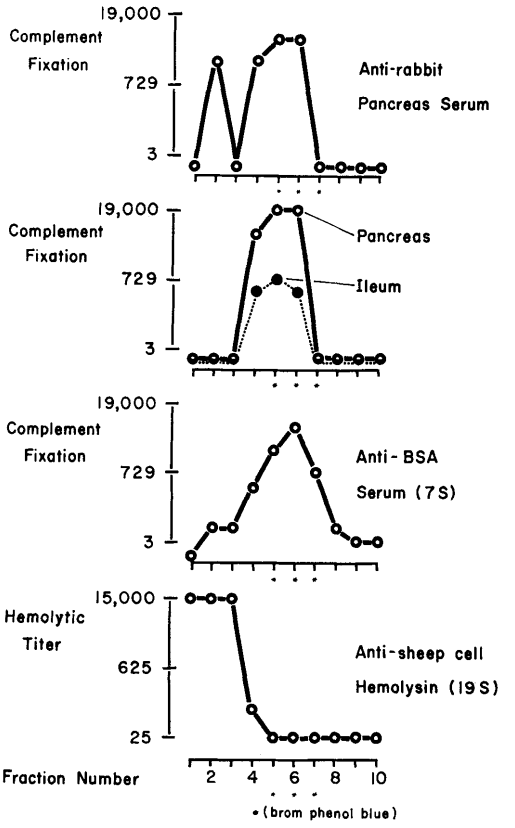

FIG. 1. Sucrose density gradient ultracentrifugation of rabbit antisera. In all graphs fraction #1 is taken from bottom of ultracentrifugation tube. From top to bottom graphs depict the following centrifugation patterns: 1. Reaction of anti-rabbit pancreas serum taken 3 weeks after initial immunization with rabbit pancreas antigen. 2. Reaction of hyperimmune anti-rabbit pancreas serum with rabbit pancreas and ileum antigens. 3. Reaction of hyperimmune rabbit anti-BSA with BSA antigen. 4. Reaction of hyperimmune rabbit anti-sheep cell hemolysin with sheep red blood cells.

7S fraction that had been isolated by sucrose density gradient ultracentrifugation. Rabbit anti-sheep red blood cell hemolysin (19S antibody) and rabbit anti-BSA (7S antibody) controls were included in the experiments.

As can be seen in Table I, when tested with pancreas extract, the reduced and alkylated pancreas antisera were negative. Reaction of the same antisera with ileum extract was also destroyed. The serological reactivity of the 7S fraction of one pancreas antiserum (779), prepared by zonal ultracentrifugation was inactivated by reduction and alkylation. An anti-BSA rabbit serum, treated in the

TABLE I. Complement Fixation Test. Reaction of untreated and mercaptoethanol-treated anti-rabbit pancreas rabbit sera with pooled rabbit pancreas.

	Antiserum	Titer* Untreated	2-Met
779	Anti-rabbit pancreas	32	0
1077	*Idem*	128	0
2580	"	16	0
2581	"	32	0
2582	"	16	0
2585	"	16	0
779	Anti-rabbit pancreas (7S fraction)	16	0
2083	Anti-BSA	128	128
	Anti-sheep red blood cell	15,000	>25

* Greatest dilution of antiserum providing 50% inhibition of hemolysis. Optimal antigen dilution used, as determined by a previous antigen dilution test.

0 indicates no reaction with a 1:2 dilution of antiserum.

† Treated with 0.1 M 2-mercaptoethanol as described in text.

same manner and tested with BSA, was unaffected, whereas the sheep cell hemolysin was inactivated.

Table II presents the findings of PCA tests carried out on mercaptoethanol-treated and untreated sera. The reduction is given in millimeters of diameter of the dyed sera around the site of injection. Injection of dye and saline and controls of normal rabbit serum did not produce a reaction at the antiserum injection site. The mercaptoethanol-treated 7S serum component, as well as the treated whole sera, showed an appreciable drop in titer when compared to the corresponding untreated sera. There was no difference in the PCA titers of the treated and untreated anti-BSA sera.

Immunoelectrophoresis was carried out on native and reduced-alkylated rabbit antisera. Following electrophoresis, the lines of precipitate were developed with the appropriate dilution of antigen. As shown in Fig. 2, untreated sera displayed one thin line of precipitate in the "fast gamma" or γ1-globulin region. Treatment with mercaptoethanol destroyed the visible precipitating ability of the globulin in its role *as antibody*. On the other hand, the pattern of precipitation of the rabbit antiserum by anti-rabbit-serum goat serum was not changed by reduction and alkylation, indicating that the γ1-globulin acting *as antigen* was not affected. The rabbit anti-BSA sera showed strong lines of precipitation in the γ1 region in both untreated and mercaptoethanol-treated samples.

Analytical ultracentrifugation of untreated and 2-mercaptoethanol-treated 7S γ globulin fraction of anti-rabbit pancreas rabbit serum was performed as a means of comparing the effect of reduction and alkylation on the sedimentation rate of the 7S fraction. The 7S component of both the untreated and treated anti-BSA sera was run as a control. In all samples, the major fraction sedimented at approximately 7S. This finding indicates that the reduced and alkylated 7S globulins of anti-rabbit pancreas rabbit serum were altered little, if at all, in their sedimentation properties even though the serological activity was destroyed by the same treatment.

Discussion. Ovary *et al*(6) and Block *et al*

TABLE II. Passive Cutaneous Anaphylaxis. PCA reaction of untreated and mercaptoethanol-treated anti-rabbit pancreas rabbit sera with pooled rabbit pancreas extract, and anti-BSA rabbit serum with BSA.

	Antisera										
	Anti-rabbit pancreas								Anti-BSA		
	779-7S fraction		2580		2581		2582		2083		
Dilution of antiserum	Untreated	2-Me	Untreated	2-Me	Untreated	2-Me	Untreated	2-Me	Untreated	2-Me	Normal rabbit serum
1:5	27	7	15	8	8	6	14	8	18	20	—
1:25	7.5	—	10	—	5	—	12	6	12	12	—
1:125	4	—	—	—	5	—	9	—	12	12	—
1:625	—	—	—	—	—	—	7	—	10	9	—

Figure given represents diameter (millimeters) of the blue spot at site of Ab injection. 2-Me = mercaptoethanol-treated.

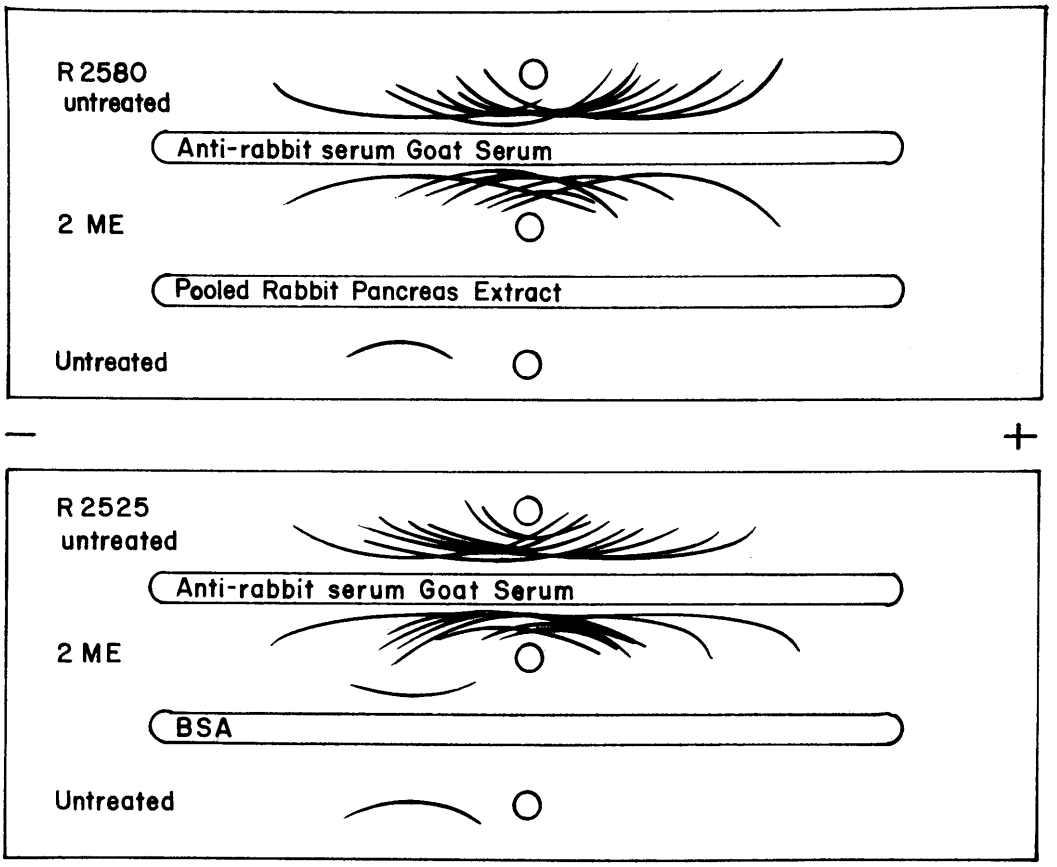

FIG. 2. Immunoelectrophoretic analysis of untreated and mercaptoethanol-treated rabbit antisera. Top slide depicts anti-rabbit pancreas serum 2580; bottom slide depicts anti-BSA serum 2525.

(7) described the serological properties of guinea pig 7S γ1 and γ2 antibodies. Guinea pig 7S γ1 antibodies, with a faster electrophoretic mobility, were found to transfer PCA to the guinea pig. The more slowly migrating 7S γ2 antibodies were shown to fix complement in the presence of antigen. Since these studies, there have been an increasing number of reports as to the effect of reducing agents on the activity of 7S γ1 and γ2 globulins from various species(8-12). Recently, Onoue *et al* (13) have reported a rabbit anti-p-azobenzenearsonate 7S γ1 antibody that loses its skin sensitizing, but not antigen-binding activity, upon reduction and alkylation.

In the present study an antibody globulin was demonstrated in rabbit sera which migrated electrophoretically as a γ1 globulin and sedimented as a 7S globulin. It was responsible for all or most of the complement-fixing, precipitating, and anaphylactic activity of the antiserum. From the results presented here it is apparent that the reduced anti-rabbit pancreas rabbit sera tested lost entirely their ability to fix complement, while the majority showed extensive reduction of skin sensitizing or anaphylactic ability and loss of measurable precipitin activity. Thus there is some correspondence between complement-fixing, anaphylactic and precipitation abilities. On first analysis, all of these functions may be ascribed to a single antibody or group of antibodies. Where higher precipitation and anaphylactic titers were found, greater resistance to reduction by mercaptoethanol was observed, even though considerable reduction in titer did occur. In these instances, it is possible that the residual serological activity is attributable to a second, mercaptoethanol-resistant globulin.

Antibodies reactive with ileum extract were also localized in the 7S fraction, and were inactivated by treatment with mercaptoethanol. These antibodies of the Kidd-Friedewald type are heat labile and are found in all adult rabbit sera. Their titer is raised somewhat by injection of rabbit pancreas extract, even though the pancreas extract exhibits little serological reaction with these antisera(2). Presumably the antigen is present in a pancreas only in small amounts. It is also found in most other organs of the rabbit. The mercaptoethanol sensitivity of this autoantibody as well as the pancreas-specific isoantibody stands in contrast with the stability of antibody to BSA when treated under the same conditions.

If antisera were taken early in the course of immunization, mercaptoethanol-sensitive antibodies were found only in the 19S fraction. While more extensive investigations are indicated, these results suggest that the immunoglobulin class of the antibody depends to some extent upon the nature of the immunizing stimulus.

The question arises as to the mechanism of action of the 0.1 M mercaptoethanol on the 7S antibody molecule. Since there was no change in the sedimentation coefficient, the molecule was probably not split. Moreover, the ability of the globulin to precipitate with goat antiserum was apparently not decreased by mercaptoethanol reduction, indicating little significant alteration in molecular configuration. Adler(12) has hypothesized that reduction weakens the structural rigidity of bivalent antibody molecules by breaking at least one disulfide bond of the antibody "backbone."

Experiments carried out on a 5S fragment of rabbit 7S γ-globulin support this view(14-16). This fragment, which was obtained by treatment of the 7S molecule with pepsin and corresponds to Fragments I and II of Porter, was treated with a reducing agent and titrated for free -SH groups. It was found that Fragments I and II of Porter were linked by one highly labile disulfide bond which is important for antibody activity. Since these fragments also contain the H chains, which are known to participate in the antibody combining sites, it is plausible that reduction of this labile bond could influence the reactivity of the antibody combining site without greatly altering the sedimentation coefficient or stereochemical configuration of the whole molecule. Some authors(14,15) have noted a loss of precipitating ability upon reduction of this bond in the 5S fragment, while others(16) have described a loss of complement fixing activity. The actual properties lost seem to depend upon the particular antibody in question. In the case of the rabbit antibody to rabbit pancreas, reduction of this highly reactive disulfide bond leads to a total loss of complement fixing ability and greatly diminished anaphylactic and precipitating activity.

The presence of mercaptoethanol-sensitive, γ1, 7S immunoglobulins has been described in rabbit antisera. They bear some similarity to the γA globulin in human sera. Grey(10) has described the presence of mercaptoethanol-sensitive antibodies resembling the γ1-globulins in the duck and in invertebrates. He has suggested that the mercaptoethanol-sensitive 7S γ-globulin represents a type of antibody that, in the evolution of immune responses, has become largely superseded in the mammal by the mercaptoethanol-resistant 7S γ-globulins. Such a hypothesis becomes especially interesting when one questions why the rabbit should produce a mercaptoethanol-sensitive antibody to rabbit organ antigens.

Summary. Sucrose density gradient ultracentrifugation has shown that anti-rabbit pancreas rabbit isoantibodies could be classed with the 7S globulins. In agar electrophoresis, the anti-rabbit pancreas rabbit antibody had a mobility of γ1 globulin. These antibodies were sensitive to reduction by 0.1 M mercaptoethanol. Reduction with mercaptoethanol and alkylation with iodoacetamide did not alter their sedimentation coefficient or precipitability by goat antiserum. There was, however, a total loss of complement fixation ability, and marked reduction in both passive cutaneous anaphylactic and precipitating abilities. The degree of reduction of these two abilities seemed to depend upon the height of the titer before treatment. The greater the titer, the more residual antibody after reduction. Naturally occurring antibodies to gastro-

intestinal mucosa also sedimented as 7S globulin and were inactivated by mercaptoethanol. 7S antibody to bovine serum albumin was mercaptoethanol resistant.

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Altered Embryonic Effects of Uterine Vascular Clamping in the Pregnant Rat by Uterine Temperature Control.* (31717)

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Brent and Franklin demonstrated that congenital malformations are produced by temporarily clamping the uterine blood supply in the pregnant rat(1). The results of this procedure vary with the duration of clamping and the stage of gestation. It is presumed that the malformations result from an altered metabolic environment within the clamped uterus. If this is true, the temperature of the tissue might influence the results of the uterine vascular clamping procedure.

This paper describes the modifying effect of controlling the temperature of the uterus of the pregnant rat upon the growth retarding, lethal, and malforming effects of uterine vascular clamping.

Materials and methods. Rats 8 days pregnant were anesthetized with pentobarbital taped in a supine position. Pregnancy had been determined by vaginal smear; 9 a.m. on the day sperm were seen was considered to be 0 hours and 0 days of gestation. After an abdominal incision, the uterine horns were brought to the exterior. The position and condition of the implantation sites were recorded. By incising the avascular areas of the mesometrium a wedge of insulating plastic could be inserted under the uterine horns between the ovarian and cervical ends. Thermister probes were placed in the rectum, between the scapulae, and beneath the clamped uterine horn. An electronic thermometer was used to record the temperature (Fig. 1).

Control of the uterine temperature was achieved by means of a thin polyethylene bag connected by tubing to a pump and a constant temperature bath. When the uterus reached the desired experimental temperature, the covering bag was removed to permit clamping of the cervical and ovarian ends of one horn. Following application of the clamps, the bag was replaced over both uterine horns. The distention of the polyethylene bag was controlled by raising or lowering a reservoir bottle outfitted with a discharge tube. The bag was sufficiently flexible to cover both

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