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Studies in Cryo-Immunology

II. TISSUE AND SPECIES SPECIFICITY OF THE AUTOANTIBODY RESPONSE AND COMPARISON WITH ISO-IMMUNIZATION

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Summary. Antibodies produced as a result of experimental cryosurgery were shown to be highly specific with regard to the tissue frozen and the species of origin. As a consequence of destructive freezing in the coagulating gland and seminal vesicle of the rabbit, antibody is produced which reacts with extracts of the seminal vesicle, coagulating, prostate and bulbo-urethral glands, but fails to react with rabbit serum or with extracts of testis, kidney, liver, thyroid, vagina, spleen, stomach, epididymis or synovium of the rabbit. It also fails to react with prostatic or related tissue of human, guinea-pig and rat. This spectrum of tissue and species specificity, along with the proof that this antibody is an autoantibody, indicates that the same antigenic material is responsible for this antibody response as was responsible for antibodies elicited by iso-immunization by injection of tissue extracts with complete Freund's adjuvant. Additional proof was obtained of this identity by use of purified fractions of rabbit prostatic tissue. Only one of the fractions was active with an antiserum produced by iso-immunization, and this same fraction was active with an antiserum produced by cryo-immunization.

Although the same autoantigen seems to be involved, the autoantibody response to the freezing of tissue is initiated much more quickly, and rises to a maximum in a much shorter period, than the analogous autoantibody response that follows repeated intradermal injections. The cryo-immunization response is generally at a maximum within 7–10 days, whereas stimulation by injection requires a period of many weeks of repeated injections, before a similar level of antibody is reached.

INTRODUCTION

In the first paper of this series (Yantorno, Soanes, Gonder and Shulman, 1967), it was shown that the freezing of tissue within the animal, as accomplished by means of liquid nitrogen, constituted an antigenic stimulus. In that study, the freezing treatment was applied to rabbit male accessory tissues of reproduction. The resultant antibody response reached a maximum level at 7 or 10 days, and then quickly fell off in titre, persisting at low level in many instances. The previous study also showed that the antibodies formed were in fact autoantibodies.

Earlier investigations on antibodies elicited by iso-immunization with accessory gland material showed that autoantibody was produced which had a high degree of specificity for the organs of this anatomical group (Shulman, Yantorno, Barnes, Gonder, Soanes

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and Witebsky, 1965; Shulman, Yantorno, Soanes, Gonder and Witebsky, 1966). Although preliminary efforts were made at that time to achieve the isolation of the autoantigen involved, no data were presented regarding purification.

It was clear that the antibody response elicited by cryosurgery should also be explored with regard to tissue and species specificity. In addition, an effort was made to determine whether the same *autoantigen* was involved as elicited antibodies following auto- and iso-immunization with extracts of accessory gland tissues.

MATERIALS AND METHODS

Antisera from cryosurgery and other procedures

The surgical methods and the application of freezing by liquid nitrogen, as well as the monitoring and control systems, have been described in the preceding paper (Yantorno *et al.*, 1967). The antisera chosen for further analysis and discussion in the present report have also been described, and are taken from the second and third of the three groups reported previously. In addition, antisera were prepared by iso-immunization with extracts of rabbit male accessory gland tissue or, alternatively, rabbit coagulating gland preparations. Antisera were also prepared in guinea-pigs against antigens of rabbit male accessory glands and were subsequently absorbed with lyophilized preparations of rabbit serum. These preparations have already been described in detail (Yantorno *et al.*, 1967).

Serological procedures

The various antisera were evaluated by tanned cell haemagglutination, by inhibition of tanned cell haemagglutination, and by immunodiffusion. These various procedures have been described (Yantorno *et al.*, 1967).

Chromatographic fractionation

The cellulose anion exchanger, DEAE-cellulose, was prepared in large batches by washing it on a Buchner funnel with four to five portions each of 0·1 N NaOH and distilled water, used alternately. The volumes were always sufficient to give a loose slurry. The absorbent was rinsed with the initial buffer and poured as a slurry into the chromatographic column in the cold room at 4°. Column dimensions of 2·5 cm in diameter by 15 or 30 cm in height were used, depending on the size of the sample. The column was packed and the contents allowed to equilibrate with the initial buffer until the effluent acquired the correct pH.

After application of the sample, fractions were collected by stepwise elution, using a GME time-activated fraction collector, model T-10. Fraction size (approximately 5 or 6 ml) was determined by convenience. The use of Mariotte bottles (of polyethylene) provided constant volumes throughout the use of a single buffer. Slight volume changes were encountered with each change of buffer.

Phosphate buffers of decreasing pH and increasing ionic strength, as described by Sober and Peterson (1958) were used, with some variations. Buffer changes were made after the optical density (at 280 mμ) of each series of fractions fell to a low value. Readings were taken on every fifth fraction, except in the region of an optical density maximum, in which every fraction was examined.

RESULTS

The antisera that were obtained as a result of cryosurgery were compared with antisera obtained from a number of injected animals. Serum R 249 served as an example

of the cryosurgery type, and three other kinds of antiserum were studied. As may be seen in Table 1, a guinea-pig antiserum to rabbit accessory tissue showed the highest titre, 1:129,072, even after complete absorption of antibodies directed against rabbit serum. R 219 and R 2212 are representatives of the group of iso-immunized rabbits. It is interesting to note that the titres shown by R 249b (7 days after cryosurgery) and R 2212c are quite similar, even though rabbit 2212 had been strongly immunized for about 14 weeks, receiving four injections totalling 30 mg of protein, whereas the 249 bleeding was made after only 1 week.

TABLE 1

COMPARISON OF TANNED CELL HAEMAGGLUTINATION TITRES OF SERA OBTAINED FROM ANIMALS SUBJECTED TO VARIOUS TYPES OF IMMUNIZATION OR CRYOSURGERY, AS TESTED WITH HUMAN ERYTHROCYTES COATED BY RABBIT MALE ACCESSORY GLAND EXTRACT AT APPROXIMATELY 0·1 PER CENT PROTEIN CONCENTRATION

Dilution of antiserum	R 249b: 7 day post-cryosurgery serum	R 2212c anti-rabbit-coagulating gland serum	R 219g anti-rabbit male accessory gland serum	Guinea-pig 12 anti-rabbit male accessory gland serum*
1:2	++	++	+	++
1:4	++	++	++	++
1:8	++	++	++	++
1:16	++	++	++	++
1:32	+	++	++	++
1:64	+	++	++	++
1:128	+	+	++	++
1:256	−	−	++	+
1:512	−	−	++	+
1:1024	−	−	++	+
1:2048	−	−	++	+
1:4096	−	−	+	+
1:8192	−	−	−	+
1:16,384	−	−	−	+
1:32,768	−	−	−	+
1:64,536	−	−	−	+
1:129,072	−	−	−	+
1:258,144	−	−	−	−

* Absorbed with 200 mg lyophilized rabbit serum/ml of antiserum.

SPECIFICITY STUDIES

The sera of two rabbits (249 and 269), obtained 7 days after cryosurgery, were tested in passive haemagglutination against cells coated with extracts of several rabbit organs. They both showed fairly strong reactions (1:512) against coagulating gland extract, while being completely negative with kidney, liver, testis, epididymis and serum. Spleen showed a titre of 1:4, and this reaction was further studied. Pre-cryosurgery serum of R 249 and 7th day post-cryosurgery sera of R 249, R 267 and R 269 all showed titres of 1:4 when tested against a pooled spleen preparation. This reaction might have been due to a natural isoantibody; to test this hypothesis, extracts of individual rabbit spleens were used, and one out of four preparations studied with R 249b and R 267b was negative. The reaction seems to be quite independent of that shown by accessory glands, for while the pre-cryosurgery serum (of R 249) gave the same titre (1:4) with spleen as the post-cryosurgery bleeding, the pre-cryosurgery serum was completely negative with accessory glands, and the post-cryosurgery serum in each case gave a definite titre. These observations

on specificity were confirmed by studying inhibition of the reaction of the 7-day serum from R 269 against cells coated by rabbit coagulating gland extract. As was expected, a coagulating gland preparation gave strong inhibition. An extract of total accessory glands was also a fairly good inhibitor, which is not surprising, since the rabbit male accessory system is a constellation of glands which includes the coagulating gland. All of the other extracts used as inhibitors showed completely negative results. These included spleen, vagina, kidney, liver, testis and serum. The negative result with spleen in this test is especially important, however, because it confirms our interpretation that the positive reaction of this organ in (direct) passive haemagglutination is completely unrelated to that shown by coagulating gland.

Further studies on the tissue specificity of such antisera were carried out with antiserum 277d, the 16-day post-cryosurgery serum that had shown the highest titre. This serum was strongly positive with coagulating gland extract (1:1024), and was negative with testis,

TABLE 2

INHIBITION OF TANNED CELL HAEMAGGLUTINATION REACTION OF R 277d (16 DAYS POST-CRYOSURGERY) SERUM AT 1:64 DILUTION (16 UNITS), AS TESTED WITH HUMAN ERYTHROCYTES COATED WITH RABBIT COAGULATING GLAND EXTRACT 9 AT ABOUT 0·1 PER CENT PROTEIN CONCENTRATION, BY THE USE OF VARIOUS RABBIT TISSUE EXTRACTS

Dilutions of inhibitors listed in the columns	Initial protein concentration of inhibitors: approximately 1 per cent									
	Coagulating gland pool 15	Seminal vesicle pool 7	Prostate pool 7	Bulbo-urethral pool 10	Liver pool 5	Thyroid pool 3	Spleen pool 3	Kidney pool 4	Vagina pool 1	Testis pool 3
1:4	–	–	–	–	++	++	++	++	++	++
1:8	–	–	–	–	++	++	++	++	++	++
1:16	–	–	–	–	++	++	++	++	++	++
1:32	–	–	–	–	++	++	++	++	++	++
1:64	–	–	–	–	++	++	++	++	++	++
1:128	–	–	–	–	++	++	++	++	++	++
1:256	–	–	–	–	++	++	++	++	++	++
1:512	–	+	–	–	++	++	++	++	++	++
1:1024	–	+	–	–	++	++	++	++	++	++
1:2048	–	+	+	+	++	++	++	++	++	++
1:4096	–	+	+	+	++	++	++	++	++	++
1:8192	–	++	+	++	++	++	++	++	++	++
1:16,384	+	++	++	++	++	++	++	++	++	++
1:32,768	+	++	++	++	++	++	++	++	++	++

kidney, liver, thyroid, vagina, spleen, stomach and serum, as tested by both haemagglutination procedures. Furthermore, separate extracts of the four individual accessory tissues—seminal vesicle, coagulating, prostate and bulbo-urethral glands—were effective in inhibition, as shown in Table 2. In immunodiffusion experiments, further confirmation was obtained of the tissue specificity of this antiserum. Extracts of rabbit kidney, spleen, thyroid, stomach, liver, vagina, testis and epididymis were all negative, when tested at both high (1·5–3·0 per cent) and low (0·2 per cent) concentration. Rabbit coagulating gland, on the other hand, gave a line of precipitation.

Studies of species specificity were also made. With antiserum R 277d tested against rabbit extract, inhibition tests revealed a high degree of species specificity, in that the accessory tissue of human, guinea-pig and rat were all negative as inhibitors, while rabbit material was (again) strongly positive. The same conclusion was also reached by direct haemagglutination. Immunodiffusion studies were made with this antiserum, as

an additional comparison; rat, guinea-pig and human prostate preparations were completely negative, whereas rabbit coagulating gland gave a definite line of precipitation.

FRACTIONATION STUDIES

In order to obtain further information concerning the immunochemical identity of the antigen active in cryosurgery, and the antigen active in iso-immunization with Freund's adjuvant, chromatographic fractions of rabbit male accessory gland extract were isolated from a column of DEAE-cellulose, as illustrated in Fig. 1. The peaks were numbered and pooled according to the sequence of buffers employed. Gel diffusion

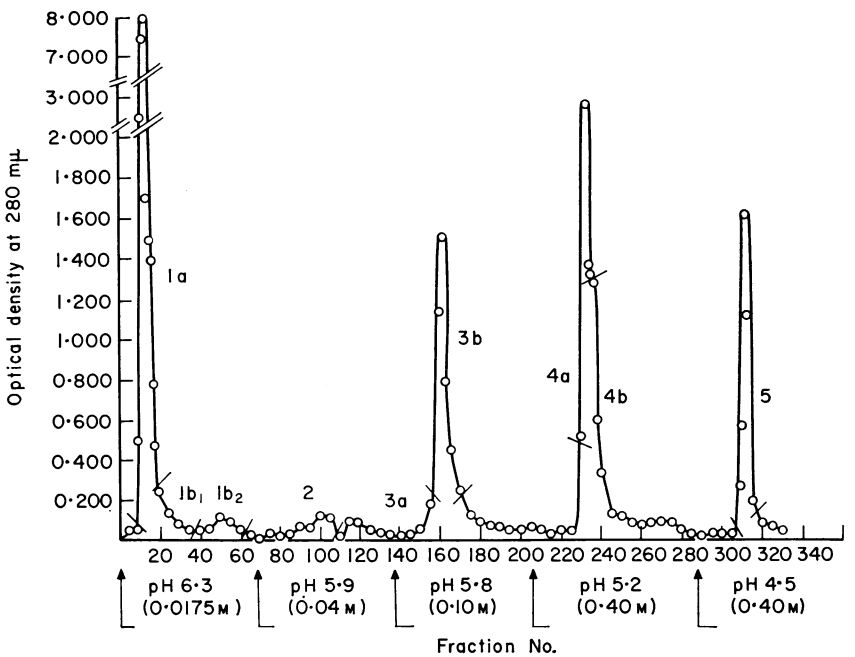

FIG. 1. Elution pattern from DEAE-cellulose chromatography of rabbit male accessory gland saline extract. The column size was 2·5 × 14·5 cm, corresponding to 7·2 g of DEAE-cellulose. The sample was applied as 5·0 ml of a 7·0 per cent solution (0·35 g) in 0·0175 M sodium phosphate buffer, pH 6·3, and a stepwise gradient employed using successive buffers, as indicated. The short lines indicate the fractions pooled to make the designated peaks.

studies were made, using a guinea-pig antiserum (GP 2) as well as a rabbit autoantiserum (R 219). Some of the results are shown in Fig. 2. Tested against the absorbed guinea-pig serum, total extract showed four lines of precipitation, whereas peaks 1a, $1b_1$ and $1b_2$ each showed a single line. It is clear that an antigenic component is present in peaks $1b_1$ and $1b_2$ that differs from the one in peak 1a. What is important is the result seen with R 219, since the reaction with this antiserum identifies the autoantigen. The reaction with peak 1a is very convincing in this respect, and furthermore one can readily identify the autoantigen among the group of antigens in the total extract. Other lines of precipitation were shown in other plates by peaks 3b, 4a and 4b, whereas peaks 2, 3a and 5

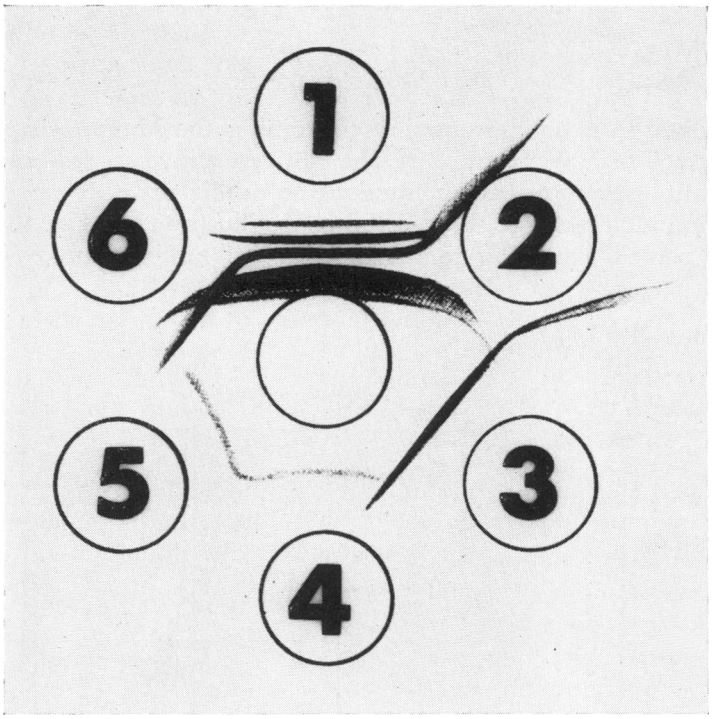

FIG. 2. Double diffusion gel precipitation. Centre well: Guinea-pig anti-rabbit prostate gland serum (GP 2), absorbed with rabbit serum. Peripheral wells: (1) rabbit male accessory gland extract preparation 5; (2) rabbit anti-rabbit accessory gland serum (R 219g); (3) and (6) chromatographic peak 1a; (4) chromatographic peak $1b_2$; (5) chromatographic peak $1b_1$.

TABLE 3

INHIBITORY TITRES OF DEAE-CELLULOSE CHROMATOGRAPHY POOLS AND BY WHOLE RABBIT MALE ACCESSORY GLAND EXTRACT, AS TESTED WITH R 267 (7 DAYS POST-CRYOSURGERY) SERUM, R 269 (7 DAYS POST-CRYOSURGERY) SERUM, AND R 219 ANTI-RABBIT MALE ACCESSORY GLAND SERUM, USING HUMAN ERYTHROCYTES COATED BY RABBIT COAGULATING GLAND EXTRACT 8 AT 0·1 PER CENT CONCENTRATION

Rabbit antisera	Inhibiting material					
	Fraction of accessory gland extract					Rabbit male accessory gland extract (about 1%)
	Peak 1a (0·27%)*	Peak 2 (0·083%)	Peak 3b (0·15%)	Peak 4a (0·29%)	Peak 5 (0·003%)	
R 267 (7 days post-cryosurgery) serum	32	<2	<2	<2	<2	64
R 269 (7 days post-cryosurgery) serum	128	<2	<2	<2	<2	1024
R 219 anti-rabbit male accessory gland serum	32	<2	<2	<2	<2	32

* Protein concentration.

showed no precipitation. Only peak 1a, however, contained the autoantigen. Numerous immunodiffusion studies of peak 1a all confirmed this conclusion.

The main fractions from this column were then used as inhibitors of R 219 and of two sera from the rabbits subjected to cryosurgery, in their reactions with tanned erythrocytes coated by rabbit coagulating gland extract. These data are shown in Table 3. Peak 1a gave good inhibition of haemagglutination by all the sera. None of the other peaks was inhibitory. These results strongly indicate that the antibodies elicited by cryosurgery of seminal vesicle and coagulating gland are directed against the same autoantigen (in peak 1a) as the antibodies formed in response to iso-immunization by injection of extract of rabbit male accessory glands.

DISCUSSION

In previous studies, iso-immunization (Shulman *et al.*, 1965, 1966) and auto-immunization (Yantorno *et al.*, 1967) of rabbits with saline extracts (SE) of prostatic tissue or of male accessory glands in general, incorporating Freund adjuvant, were shown to be successful in eliciting an autoantibody response, as judged by the criteria of tanned cell haemagglutination and of gel diffusion. This antibody was shown to be highly organ specific, reacting with preparations from the accessory tissues, namely, seminal vesicle, coagulating, prostate and bulbo-urethral glands, and also with seminal plasma, but failing to react with preparations from testis, bladder, kidney and a variety of other rabbit tissues. In the present studies, antibodies elicited in response to cryosurgery of the coagulating gland and seminal vesicle of the rabbit have also been shown to be directed against an antigen present in the accessory glands complex. Other reports have described the surgical procedure and temperature measurements in greater detail (Shulman, 1967; Jagodzinski, Yantorno and Shulman, 1967).

In a heterologous system, guinea-pig anti-rabbit male accessory gland serum, tested against the corresponding antigenic material, recognized more antigenic determinants of the accessory gland preparations than did the cryo-, auto- or iso-systems, and gave the highest titres in tanned cell haemagglutination. R 2212, which had been immunized with about 30 mg of pooled coagulating gland extract over a 14-week period, and R 249, which had been cryo-immunized only 1 week previously, showed similar titres, nonetheless. It has been our experience with the iso-immunization procedure that no antibody response can be detected in any injected rabbit only 1 week after the injection.

In the studies reported here, evidence for a high degree of tissue specificity has been presented, from results with several antisera formed in response to cryo-immunization. It was found that such an antiserum reacts with extracts of seminal vesicle and prostate, bulbo-urethral and coagulating glands, but fails to react with rabbit serum or extracts of testis, kidney, liver, thyroid, vagina,* spleen, stomach, epididymis and synovia. It also showed complete lack of reactivity with human, guinea-pig and rat accessory tissue. In all these particulars, it showed precisely the same spectrum of specificity as was demonstrated in the earlier studies on antiserum from iso-immunized rabbits. Similar freezing of the kidney fails to lead to any subsequent serum activity against extract of accessory glands (Shulman, Yantorno and Bronson, 1967).

It has already been demonstrated that this antigen is, in fact, an autoantigen (Yantorno

* With regard to the non-reactivity with vagina, it may be mentioned that in recent studies on *iso-immunized* rabbits, some slight reactivity has been detected between anti-accessory gland serum and vagina extract. However, this activity, even if specific, is comparable to approximately 1/1000 of the activity of a coagulating gland extract of similar total protein concentration.

et al., 1967), and it was suggested that the antibodies formed in response to cryo-immunization and those elicited by iso-immunization with pooled accessory gland extract were responses to the same antigen. Additional proof has been obtained in the present studies by comparison of the effectiveness of protein fractions from this tissue, in inhibition of haemagglutination. The active fraction 1a was proven to contain autoantigen by means of a direct test in gel diffusion with serum from R 219, an iso-immunized rabbit.

Circulating autoantibodies have been claimed to be part of the response to various forms of injury in man and animals. Complement-fixing activity has been observed shortly after liver damage induced by administration of carbon tetrachloride to rats (Weir, 1961, 1963). Although there are some similarities to our findings, notably in the speed of the response, there are striking differences. The factor described by Weir was shown to be active against liver from various species and also against a variety of other rat organs. In other words, this activity showed no tissue specificity or species specificity, and thus differs from the antibody response to the cryosurgical stimulation. Pinckard and Weir (1966) recently reported that this liver antigen is primarily associated with the mitochondrial preparation, and speculated that an animal might not be tolerant to its subcellular particulate components, and that this could explain the autoantibody response. This is an interesting suggestion that merits further exploration, including the possibility that the cryogenic stimulation also may involve a mitochondrial, or similar, component. However, there are some additional points of difference between Weir's findings and ours. The factor produced by liver injury was shown to be exclusively a 19S globulin (Weir, 1964). In contrast, recent studies in our laboratory have revealed that 19S and 7S globulins are both involved as antibody forms in the cryo-immunization response (Shulman, Bronson, Riera, Brandt and Yantorno, unpublished).

Boyden (1964) has demonstrated by haemagglutination and complement fixation methods that a distribution of titres can be found in the reaction between normal rabbit sera and extracts of rabbit skin. However, no other tissues were tested, apparently, and one cannot be sure that a tissue-specific effect was obtained. More recently, a series of detailed studies has been reported on the immune response to thermal injury. These investigations are an extension of earlier reports on burn damage and the development of a resulting anti-globulin reaction (Atherton, Merrill and McCarthy, 1960; McCarthy and McCarthy, 1963, 1964). Current studies on burn damage to the skin of rats have shown that serum agglutinating activity subsequently appears against rat erythrocytes, and this activity is interpreted as being due to autohaemagglutinins (Kano, Milgrom, Witebsky and Rapaport, 1966a, b). These studies extended to guinea-pigs and rabbits have demonstrated antibodies reacting with erythrocytes or with γ-globulin of animal species different from that of the injured animal (Kano, Milgrom and Rapaport, 1967). However, although these investigations are of considerable fundamental interest and clarify the antibody response to tissue injury resulting from the application of heat, they are not at all comparable to our studies on the antibody response to tissue injury resulting from the application of cold. This conclusion follows from the observation that the former type of antibody reacts with antigens of red blood cells, rather than those of the tissue that was damaged, and may react more readily with antigens from other species than with homologous antigens. The antibody produced by cryo-immunization is, in contrast, highly specific to the tissue damaged and to the species involved.

From our experimental data, it can be concluded that cryo- and iso-immunization elicit antibodies of very similar specificities. It has been proposed that in adjuvant-type

immunization, there are antigenic changes, caused by the Freund's adjuvant, that trigger autoimmune mechanisms (Najjar and Fisher, 1956). Weigle (1965) on the other hand, has shown that circulating antibodies to native thyroglobulin can be produced, as well as thyroiditis, in rabbits by injecting, without adjuvant, homologous thyroglobulin altered by coupling it to diazonium derivatives of arsanilic and sulphanilic acids. If the hypothesis of antigenic change is applied to our system, a requirement would be that the two different methods of immunization we have employed are able to produce similar molecular changes. However, it must be recalled that even though freezing has been reported to cause some molecular changes (Chilson, Costello and Kaplan, 1965), the general consensus is that one-step freezing and thawing is an effective mode of preservation (Pennell, 1965). Further studies of the biophysical and biochemical characteristics of the autoantigen obtained from frozen and non-frozen control tissue will be necessary to clarify this question. It should be stated that in almost all our previous iso-immunization studies, the extracts were prepared from tissue that had been frozen for some time in storage. In all the *in vitro* tests, also, antigen was prepared from tissue that had been stored frozen.

In view of these considerations, an alternative interpretation of our data is that cryosurgery releases a normally sequestered antigen directly into the bloodstream. Liberation of tissue antigens into the circulation, following surgical or chemical damage, has been described for thyroid (Hjort, 1961; Hjort and Pedersen, 1962) and for liver (Sargent, Myers and Richter, 1966). The possible factors that may be involved in the rupture of cell membranes as a result of freezing have been discussed in some detail, along with a number of observations on temperature gradients and freeze–thaw–time curves (Jagodzinski *et al.*, 1967). Cryo-immunization should be applied to other autoimmune systems, because it provides an experimental model of autoimmunity which is characterized by a quick response and perhaps a more natural one. The freezing stimulus can be considered to be an alternative to the type of procedure that involves the use of an adjuvant in injection of antigen.

Several approaches may be employed to attain a more lasting response. At present, we are exploring some possibilities. In current work, we have found that rabbits which have been iso-immunized and subsequently, as a second stimulus, subjected to cryosurgery of the seminal vesicle and coagulating gland, show a very marked response (Riera, Brandt and Shulman, unpublished). Alternatively, when rabbits which have been subjected to cryosurgery are iso-immunized, as a second stimulus, about 7 weeks afterwards, a quick and strong reaction is shown (Yantorno, Orsini and Shulman, unpublished). These results also give indirect evidence supporting the conclusion that the same autoantigen is involved. Studies are being continued on the nature of the secondary response, when cryo-immunization constitutes one of the antigenic stimulations.

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