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Restoration of the Specific Immunological Reactivity of Tolerant Rabbits by Conjugated Antigens

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Summary. Immunization of HSA-tolerant animals with sulphanil-HSA induces the formation of antibodies that react with native HSA. To test whether the anti-HSA response does actually represent complete restoration of the original reactivity, the antibodies produced following immunization of tolerant animals with sulphanil-HSA were compared to those produced by normal rabbits immunized with the native antigen. Gel-diffusion analysis of the precipitins disclosed that the patterns of the two types of antibody were not completely identical. The antibodies of the 'restored' tolerant rabbits were directed mainly towards an antigenic determinant of the native HSA, to which normal rabbits immunized with HSA did not respond. When the 'restored' rabbits were challenged with native HSA after a prolonged rest period, the antibodies formed thereafter were completely identical with anti-HSA produced by normal HSA-immunized rabbits. Thus, complete restoration of the original immunological reactivity to HSA was achieved following an intermediary stage of atypical anti-HSA elicited by the conjugated protein.

Attempts to break down natural tolerance to RSA by treatment with sulphanil-RSA failed to give any evidence of an autoimmune elimination of RSA. To test whether this inability to break tolerance to RSA was due to the presence of an excess of native protein, animals that had been made tolerant to HSA were immunized with a mixture of sulphanil-HSA and native HSA. Under such conditions, the termination of acquired tolerance was inhibited. The possible relevance of these observations to the cellular basis of immunological tolerance is discussed.

INTRODUCTION

Termination of specific immunological tolerance to protein antigens can be induced by immunizing tolerant animals with hapten-conjugates of the respective tolerogens. This has been demonstrated both in animals made tolerant by neonatal administration of HSA, BSA or BGG (Cinader and Dubert, 1955; Weigle, 1962; Schechter Bauminger, Sela, Nachtigal and Feldman, 1964), and in adult rabbits made specifically unresponsive to HSA following total body X-irradiation (Nachtigal and Feldman, 1964). In all these cases, however, breakdown of immunological tolerance was inferred from the observation that antibodies reacting with the carrier protein are produced as a result of immunization with the protein–hapten conjugate. Whether, on this apparent termination of acquired tolerance, the animals regain their original capacity to recognize immunologically the regular immunogenic determinants of the native protein or whether they recognize only the conjugated complex, is not known. In a similar system of breakdown of tolerance to protein antigens by immunization with cross-reacting proteins, it has been claimed that

the immune response obtained was directed only to the cross reacting determinants of the two antigens (Weigle, 1964). In this case, the immunological reactivation did not represent complete reversal of the tolerant state.

In the present study, experiments were carried out to test whether immunization of tolerant rabbits with sulphanil-conjugates does indeed result in a complete restoration of the original pattern of immunological recognition towards the tolerogenic protein. An analysis was made of the types of antibodies obtained following immunization of tolerant animals with sulphanil-proteins. The results were expected to clarify both the molecular factors which determine whether or not a tolerant state will be terminated and the cellular basis of tolerance.

In previous experiments, we demonstrated the identical pattern of antibodies formed in response to the immunogenic effect of azo-protein conjugates (Nachtigal and Feldman, 1964) and polypeptidyl-proteins (Schechter *et al.*, 1964) in animals either made tolerant to the protein carrier or naturally tolerant to the protein antigen. The inference was drawn that the mechanism underlying natural tolerance is most probably similar to that of experimentally induced tolerance. If this is so, the breakdown of experimentally acquired tolerance may have a bearing on the problem of autoimmune reactions. Attempts were therefore made to terminate natural tolerance of rabbits to their native albumin (RSA), using a similar approach to that which resulted in the termination of induced tolerance to HSA. The results of this study led to the analysis of the role of native protein antigen in inhibiting tolerance breakdown by its conjugate, and the possible significance of this inhibition.

MATERIALS AND METHODS

Antigens

Human serum albumin (HSA), rabbit serum albumin (RSA), ^{131}I-labelled HSA and RSA, as well as sulphanil-HSA and RSA were the same as described before (Nachtigal and Feldman, 1964).

Animals

Rabbits of both sexes from local stock, weighing between 2 and 3 kg were used.

X-ray treatment

The animals were subjected to 550 R total body X-irradiation, as previously described (Nachtigal and Feldman, 1963).

Induction of unresponsiveness to HSA

Irradiated rabbits were given 20 mg HSA intravenously 24 hours after the X-ray treatment, followed by identical 'booster' doses of HSA at fortnightly intervals. Ten weeks after irradiation, the animals were found to be responsive to administered control antigens, and, in almost all cases, unresponsive to HSA.

Serological technique

Binding antibodies were measured using Farr's technique according to the modification of Terres and Wolins (1961). Gel diffusion tests were performed in agar layered on microscopical slides, employing the standard equipment of Messrs LKB, Stockholm, Sweden.

EXPERIMENTAL

COMPARATIVE ANALYSIS OF NORMAL AND 'RESTORED' ANTI-HSA

The purpose of the initial experiments was to analyse the anti-HSA antibodies elicited by sulphanil-HSA in rabbits that had been made tolerant to the carrier protein. Previous studies on the antigenic determinants operative in the immune response to sulphanil-conjugates have demonstrated that, in addition to the haptenic specificity, such conjugates acquired a determinant which appeared to comprise the sulphanilic group and part of the carrier protein, and was thus specific to the conjugated homologous carrier (Nachtigal and Feldman, 1964). The question therefore arises whether those antibodies reacting with HSA which are produced during the breakdown of tolerance following immunization with sulphanil-HSA, are, in fact, antibodies to the regular determinants of the native protein, or whether they are directed only to 'conjugation' determinants that cross-react with the native carrier. To elucidate this problem, ten rabbits were made tolerant to HSA and then immunized with sulphanil-HSA by means of seven fortnightly injections of 17 mg conjugate each. A control group of normal rabbits was immunized with native HSA. The precipitins reacting with HSA produced in the animals made tolerant to HSA were compared to those produced in the control rabbits, by gel-diffusion analysis. The evolving pattern of precipitins revealed that the two types of antibodies were not completely identical (Fig. 1). The spurs demonstrated unexpectedly that the antibodies of the 'restored'

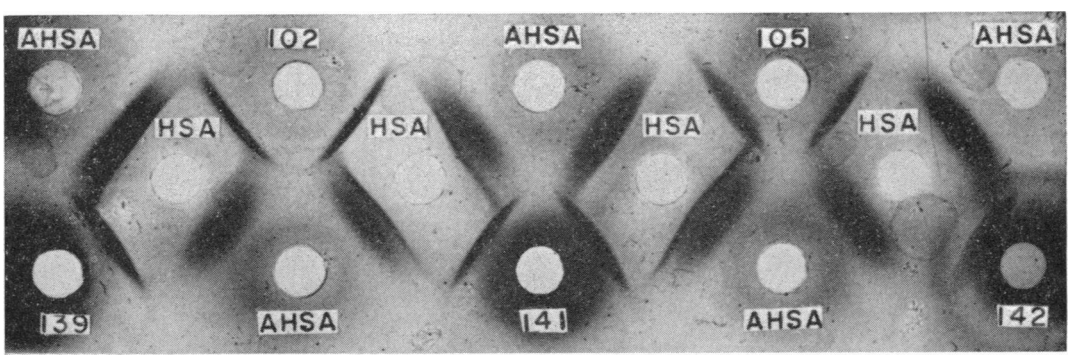

FIG. 1. Comparison of anti-HSA antibodies from normal rabbits (AHSA) and from 'restored' rabbits that had been tolerant to HSA (Nos. 102, 105, 139, 141 and 142). 'AHSA' represents a pool of anti-HSA sera.

rabbits were mainly directed against an antigenic determinant of the native HSA, to which normal rabbits immunized with native HSA did not react. This determinant was, obviously, non-immunogenic in the native HSA molecule, and acquired immunogenicity only after conjugation with sulphanilic acid. Although non-immunogenic and distinctly non-tolerogenic, it is nevertheless reactive in the native protein.

The precipitation pattern of anti-HSA produced on immunization with sulphanil-HSA disclosed, in addition to the response to 'conjugation' antigen, an anti-HSA antibody common to both normal and 'restored' rabbits. This could represent true reversal of tolerance to HSA. Experiments were therefore carried out to test whether, as a consequence of abolished tolerance, the 'restored' rabbits would in fact *recognize* the regular antigenic determinants of native HSA. Seventy-five days after the last sulphanil conjugate treatment

the 'restored' rabbits were challenged intraveneously with 20 mg of native HSA. The animals were bled periodically, and their binding titres against HSA, sulphanil-HSA and sulphanil-RSA were measured (Fig. 2). It was found that the challenge with native HSA restored the reduced binding capacity for sulphanil-proteins to its original level, while at the same time the response to unconjugated HSA was boosted to about five-fold the highest titre obtained after sulphanil-conjugate treatment. This excessive stimulation of anti-HSA response by immunization with native HSA represents a true restoration of the immunological reactivity that had been abolished in the tolerant state.

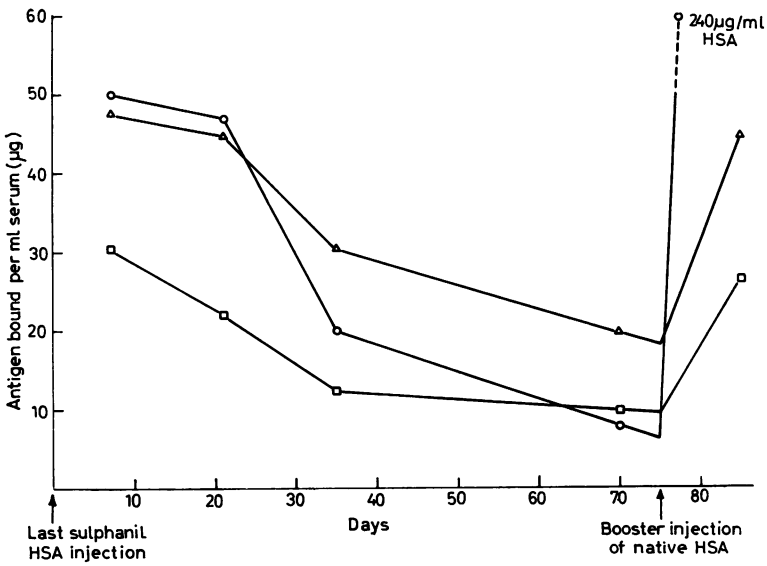

FIG. 2. Binding of HSA (○) and of sulphanil-proteins (△, sulphanil-HSA; □, sulphanil RSA) by sera of 'restored' rabbits after interruption of treatment with conjugated HSA and stimulation with native HSA.

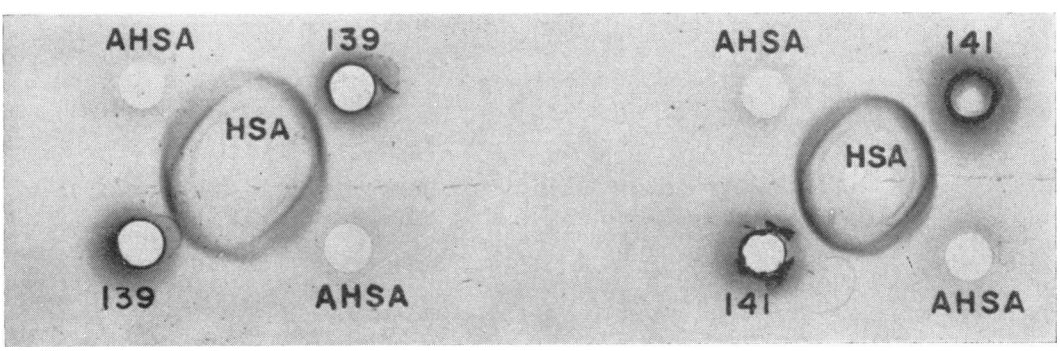

FIG. 3. Identity of the anti-HSA antibodies evoked by native HSA in normal rabbits (AHSA) and in restored rabbits after interruption of treatment with conjugated HSA (Nos. 139 and 141). 'AHSA' represents a pool of anti HSA-sera.

This conclusion was substantiated by the gel-diffusion patterns of the antisera. The anti-HSA produced in the 'restored' animals after delayed challenge with HSA is identical

with anti-HSA produced in normal HSA immunized rabbits (Fig. 3). The 'irregular' anti-HSA antibody (against 'conjugation determinants'), which decreased during the 75 days non-treatment period, was apparently not stimulated by the native antigen.

It may therefore be concluded that during the first stages of tolerance breakdown, anti-HSA antibodies formed by the HSA-conjugates are directed mainly to an antigenic determinant which is not immunogenic in the native antigen. When tested later, all 'restored' animals have the capacity to react to the ordinary immunogenic determinants of HSA, and the pattern of antibody produced in response to HSA indicates a complete restoration of the immunological recognition of the tolerogen.

ATTEMPTS AT BREAKING DOWN NATURAL TOLERANCE TO RSA BY TREATMENT WITH SULPHANIL-RSA

Our previous work has shown that anti-sulphanil-RSA produced in rabbits (the homologous animals) showed an analogous pattern of antibodies to those evoked by sulphanil-HSA in rabbits with acquired tolerance to HSA (Nachtigal and Feldman, 1964). Taking into consideration also the present finding that immunization with conjugated HSA leads to a restoration of the specific reactivity to native HSA of rabbits tolerant to this antigen, it was considered desirable to test whether continuous immunization with sulphanil-RSA will lead to the breakdown of natural tolerance to RSA.

It could be conceived that in the experiments on immunization with sulphanil-RSA (Nachtigal and Feldman, 1964), anti-RSA might nevertheless have been produced, but remained undetected by the methods employed, owing to its absorption *in vivo* by the circulating native RSA. The production of such auto-antibodies, should it occur, could be detected by comparing the immune elimination of labelled native RSA in rabbits pretreated with conjugated RSA with that of untreated normal controls.

The following experiment was therefore carried out: ten rabbits received six intravenous injections each of 17 mg sulphanil-RSA, at intervals of 14 days, and a seventh dose incorporated in Freund's incomplete adjuvant administered intramuscularly. The nine surviving animals and ten untreated controls were injected intravenously with 50 mg of radioiodinated RSA (0·5 mc ^{131}I per rabbit), 4 weeks after the last immunization. Samples of sera were taken at 2–4-day intervals, and the activity was measured in a well-type scintillation counter. Elimination curves based on the percentage elimination calculated for each group of animals were found to be practically identical (Fig. 4). Thus,

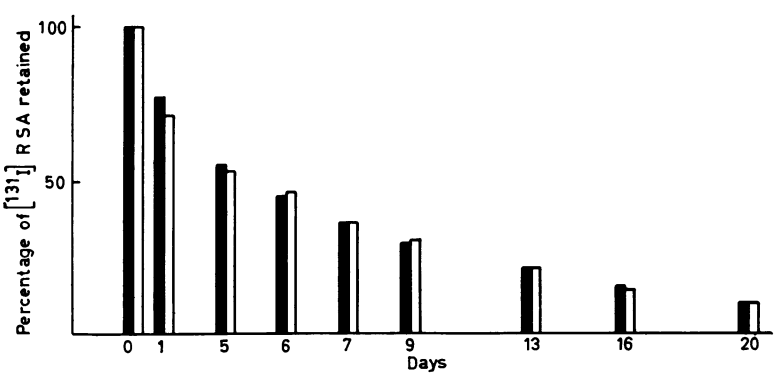

FIG. 4. Percentage of iodine-labelled RSA retained in normal rabbits (white columns) and in rabbits pretreated with sulphanil-RSA (black columns).

no evidence of an autoimmune elimination of RSA could be demonstrated in rabbits that had been immunized with sulphanil-RSA. The conjugated albumin did not reverse the state of natural tolerance of rabbits to their own serum albumin.

THE RESTORATION OF THE IMMUNE RESPONSE TO HSA IN TOLERANT RABBITS TREATED EITHER WITH CONJUGATED HSA OR MIXED NATIVE AND CONJUGATED HSA

The finding that natural tolerance is not terminated by immunization with the conjugated 'tolerogen', while acquired tolerance is, may be accounted for on the basis of two alternative suppositions: (a) natural and acquired tolerance are basically dissimilar phenomena (Boyden and Sorkin, 1962), or (b) they are essentially similar. The fact that natural tolerance was not broken down by RSA conjugates, whereas acquired tolerance to HSA was, is due to the presence of a great excess of the native RSA which might stabilize the state of tolerance; such excess of native HSA is absent in the HSA-tolerant rabbits. If the second alternative is correct, immunization of HSA-tolerant rabbits with a mixture of sulphanil-HSA and native HSA should not result in tolerance breakdown.

In order to test these hypotheses, rabbits made tolerant to HSA following X-irradiation were divided into three groups: group I (ten animals) was injected intramuscularly with 17 mg of sulphanil-HSA incorporated into incomplete Freund's adjuvant, followed by fortnightly intravenous injections of 17 mg conjugate, up to a total amount of 68 mg sulphanil-tolerogen. Rabbits of group II (twelve animals) were treated identically, except that 50 mg of native HSA was added to each dose of the conjugated protein. Group III (five animals) served as the control group, and was given parallel intravenous injections each consisting of 20 mg HSA. All animals were bled 8 weeks after the first conjugate treatment, then given one more injection of the appropriate antigen and bled again 8 days later.

The sera were tested for binding antibody to HSA and to sulphanil-conjugates of HSA and RSA (Table 1). Of nine surviving rabbits treated with the conjugated HSA alone (group I), 8 demonstrated restoration of response to HSA. This was shown by the production of binding and precipitating antibodies to HSA and by rise in titre after a booster injection of the conjugate. One animal remained unresponsive to HSA, i.e. less than 10 μg HSA/ml serum, out of 100 μg added, was bound.

TABLE 1

RESTORATION OF THE IMMUNE RESPONSE TO HSA IN RABBITS MADE TOLERANT TO HSA BY X-IRRADIATION AND TREATED THEREAFTER WITH SULPHANIL-HSA ALONE OR MIXED WITH THE NATIVE ALBUMIN

Days of treatment with conjugated HSA	HSA bound per millilitre of serum (μg)				
	Conjugated HSA alone		Conjugated + native HSA		Controls treated with native HSA
	Restored response in eight animals	Non-restored response in one animal	Non-restored response in seven animals	Restored response in two animals	
56	32±15	4·0	2±2	34±12	1±0
78	60±16	4·0	2±2	33±21	1±1

Seven out of the nine survivors of group II remained unresponsive to HSA. The remaining two rabbits of this group formed binding antibodies to HSA of an order of magnitude similar to that obtained from group I, although no precipitins could be

detected in gel diffusion tests, and there was no significant increase in titre after a booster injection. At the same time both groups of animals responded equally to the hapten moiety of sulphanilic acid (Table 2), thus demonstrating the specificity of the stabilizing action of the native tolerogen on the state of tolerance.

TABLE 2

REACTIONS WITH SULPHANIL-PROTEINS OF SERA FROM RABBITS TOLERANT TO HSA AFTER TREATMENT WITH SULPHANIL-HSA, EITHER ALONE OR IN MIXTURE WITH NATIVE HSA

Days of treatment with HSA conjugate	Sulphanil-protein bound (μg)					
	Treated with HSA conjugate alone		Treated with conjugated + native HSA		Controls treated with native HSA	
	HSA conjugate bound	RSA conjugate bound	HSA conjugate bound	RSA conjugate bound	HSA conjugate bound	RSA conjugate bound
56	51±16	32±18	46±22	40±25	2±2	7±3
78	42±4	30±8	39±4	31±13	0	0

All the rabbits from the control group III remained unresponsive.

From these results, it can be inferred that the 'breakdown' of tolerance to protein antigens in rabbits immunized with azo-conjugates of the respective proteins is inhibited if the native protein is present in the circulation, even in comparatively small amounts. These observations provide experimental support to previous statements regarding the tolerance stabilizing effect of the native tolerogen (Humphrey, 1964; Nachtigal and Feldman, 1964).

DISCUSSION

The distinction between true and apparent restoration of immunological recognition is of primary importance in the study of the cellular basis of immune tolerance. The difference is not always easy to demonstrate: it is conceivable, for instance, that in one out of two cross-reacting antigens, a common determinant may be present in a reactive but non-immunogenic form and may consequently be also non-tolerogenic. The same determinant may, however, be fully immunogenic in the other antigen. When tolerance is induced to the first antigen, the test animal will remain potentially reactive to the common determinant and will form antibodies against it in response to immunization with the cross-reacting antigen. These antibodies will react with the tolerogen, thus simulating a breakdown of tolerance, although they will obviously not represent a real restoration of a 'deleted' immunological reactivity (Weigle, 1964). This is, apparently, the situation pertaining on immunization of HSA-tolerant animals with sulphanil-HSA: the gel-diffusion analysis of the antibody response elicited by conjugated tolerogens in HSA-tolerant rabbits indicated that an important part of this response was directed against antigenic determinants of the HSA which acquired immunogenicity only following conjugation. It seems probable that the animals were not all tolerant to this determinant in the first place. If this is the case, the first stage of the apparent restoration of anti-HSA response did not represent actual reversal of the tolerant state but rather an immune reaction against antigenic components of the HSA molecule which were only made immunogenic after coupling. On the other hand, antibodies which manifested an identity pattern with the regular determinants of native HSA were also produced, but at a much lower level. This

type of antibody may represent true reversal of the tolerant state. Rabbits challenged with the native antigen after 75 days without treatment, responded to the HSA by a five-fold increase in the original titre of anti-HSA antibodies. Thus, following the immunogenic effect of the 'conjugation' determinant of HSA, the previously tolerant animals also regained their capacity to recognize the native HSA, and produced antibodies which showed an identical specificity to those formed in normal rabbits immunized with the same antigen. It is, therefore, concluded that the tolerant state was completely reversed. Whether the immunogenic effect of a 'conjugation' antigen is a prerequisite which determines whether or not there is complete termination of tolerance is an intriguing question which must await further testing.

This finding can hardly be reconciled with the clonal selection concept of tolerance. If tolerance to HSA is considered to originate in the removal of clonal stem cells, which were predetermined to form only anti-HSA, it must consequently be assumed that the protein conjugate directs a specific mutation which carries a gene for the original anti-HSA. It would appear more likely that cells which had been previously non-reactive to HSA regain reactivity due to this treatment. Accordingly, the immunogenic conjugate and the native HSA seem to have both acted on the same target cell. From this one would predict that in an animal tolerant to HSA, the native tolerogen and the immunogenic sulphanil-HSA would compete for the same cellular site. This is, in fact, strongly suggested by the immunization experiments of tolerant animals with a mixture of conjugated and native HSA. Under such conditions, i.e. in the presence of the native tolerogen, the azo conjugate failed to induce tolerance breakdown. This may indicate a state of competitive inhibition between the two components, pointing to the existence of a common cellular target.

Since native HSA inhibited the breakdown of acquired tolerance, assuming that natural tolerance and experimentally induced tolerance are based on the same mechanism, it would be expected that attempts to terminate natural tolerance to RSA, i.e. to produce an autoimmune response to RSA by injecting sulphanil-RSA, would fail. The experiments, reported in the present study demonstrate such failure; however, this does not necessarily imply that autoimmune reactions to other tissue components may not be elicited through a similar mechanism. This may apparently happen in cases in which the amount of circulating native antigen is very low. Thus, Pokorna and Vojtiskova (1964) demonstrated that an autoimmune aspermatogenesis could be evoked in guinea-pigs when the latter were treated with testicular antigens conjugated with sulphanilic acid. Weigle (1965) has likewise induced an autoimmune response to altered homologous thyroglobulin, to which natural tolerance may perhaps exist (Hjort and Pedersen, 1962).

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REFERENCES

BOYDEN, S. V. and SORKIN, E. (1962). 'Effect of neonatal injections of protein on the immune response, to protein–hapten complexes.' *Immunology*, **5**, 370.

CINADER, B. and DUBERT, J. M. (1955). 'Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin.' *Brit. J. exp. Path.*, **36**, 515.

HJORT, T. and PEDERSEN, G. T. (1962). 'Thyroid antibodies and thyroglobulin in the serum.' *Lancet*, **ii**, 259.

HUMPHREY, J. H. (1964). 'Immunological unresponsiveness to protein antigens in rabbits.' *Immunology*, **7**, 449.

NACHTIGAL, D. and FELDMAN, M. (1963). 'Immunological unresponsiveness to protein antigen in rabbits exposed to X-irradiation or 6-mercaptopurine treatment.' *Immunology*, **6**, 356.

NACHTIGAL, D. and FELDMAN, M. (1964). 'The immune response to azo-protein conjugates in rabbits unresponsive to the protein carriers.' *Immunology*, **7**, 616.

POKORNA, Z. and VOJTISKOVA, M. (1964). 'Autoimmune damage of the testes induced with chemically modified organ specific antigen.' *Folia biol. (Praha)*, **10**, 261.

SCHECHTER, I., BAUMINGER, S., SELA, M., NACHTIGAL, D. and FELDMAN, M. (1964). 'Immune response to polypeptidyl proteins in rabbits tolerant to the protein carriers.' *Immunochemistry*, **1**, 249.

TERRES, G. and WOLINS, W. (1961). 'Enhanced immunological sensitization of mice by the simultaneous injection of antigen, and specific antiserum.' *J. Immunol.*, **86**, 361.

WEIGLE, W. O. (1962). 'Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens.' *J. exp. Med.*, **116**, 913.

WEIGLE, W. O. (1964). 'The immune response of BSA tolerant rabbits to injections of BSA following the termination of the tolerant state.' *J. Immunol.*, **92**, 791.

WEIGLE, W. O. (1965). 'The induction of autoimmunity in rabbits following injection of heterologous or altered homologous thyroglobulin.' *J. exp. Med.*, **121**, 289.