IMMUNE RESPONSE TO CHEMICALLY MODIFIED FLAGELLIN

I. INDUCTION OF ANTIBODY TOLERANCE TO FLAGELLIN BY ACETOACETYLATED DERIVATIVES OF THE PROTEIN

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One of the basic questions of immunology is what makes a good antigen? Why is it that some substances, such as the serum proteins, are comparatively poor at eliciting antibodies, whereas other substances, such as the bacterial proteins, are very good antibody inducers? From data presented by numerous workers it appears that for a substance to induce antibody formation two general properties are required. Firstly, the substance must possess chemical groupings (antigenic determinants) which are regarded as "foreign" by the injected animal, and secondly, the substance must be capable of stimulating the animal to produce antibodies against these foreign determinants (see reference 1). At present much is known about the physical and chemical nature of many antigenic determinants, particularly those of the simpler antigens, such as the carbohydrates (2–4) and synthetic polypeptides (1, 5, 6). In contrast, little is known about those factors which enable an antigenic determinant to stimulate antibody formation.

Work in this laboratory has been directed towards determining some of the factors which influence the immunogenicity of antigens using the globular protein flagellin from *Salmonella adelaide* organisms as an antigen model. Flagellin (mol. wt. 40,000) has the advantage that it is highly immunogenic, injections of as little as 10 ng in saline into rats producing detectable amounts of anti-flagellin antibody (7). On the other hand, partial degradation of the molecule with cyanogen bromide drastically reduces the antibody-forming capacity of the protein and produces a preparation with strong tolerance-inducing properties (8, 9). Data presented in this paper demonstrate that by a process of acetoacetylation, even stronger tolerance-inducing preparations of flagellin can be obtained. Some preliminary aspects of this work have been reported elsewhere.[1]

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[1] Parish, C. R. 1971. Suppression of antibody formation and concomitant enhancement of cell-mediated immunity by acetoacetylated derivatives of *Salmonella* flagellin. *In* Immunolgoical Tolerance to Microbial Antigens. H. Friedman, editor. *Ann. N.Y. Acad. Sci.* In press.

Materials and Methods

Animals.—Randomly bred Wistar rats of 6–10 wk of age and of either sex were used. Animals were obtained from a Wistar rat colony established from breeders supplied by the Walter and Eliza Hall Institute of Medical Research, Parkville, Australia. In all cases rats were bled from the tail.

Antisera against flagellin were raised in 6-month-old New Zealand rabbits.

Antigens.—*Salmonella adelaide* flagellin (strain SW1338; H antigen, fg; 0 antigen, 35) was prepared as reported elsewhere (10). A cyanogen bromide (CNBr) digest of *S. adelaide* flagellin was obtained as described previously (11). All injections were in saline into the hind footpads (50 μl/footpad).

Antisera to Flagellin.—Antisera were raised in rabbits by initially injecting 0.5 mg of flagellin emulsified in Freund's complete adjuvant (FCA)[2] at multiple, subcutaneous sites. Four wk later animals were challenged in a similar manner with 0.5 mg of flagellin emulsified in Freund's incomplete adjuvant (FIA). Then, 2 wk later, the rabbits were injected intravenously with 0.5 mg of flagellin in saline. All rabbits were bled 7–14 days after the final challenge. The antigenic studies presented in this paper were performed with antiserum (single bleed) from one hyperimmune rabbit. However, comparable results were obtained if other rabbit antisera were employed.

Conditions for Acetoacetylation of Flagellin.—*S. adelaide* flagellin was acetoacetylated with diketene (acetoacetic anhydride) according to a previously described method (12, 13). Flagellin (5 mg/ml) in 0.04 M borate-boric acid buffer, pH 8.5, was reacted with a 19–960 M excess of diketene (K & K Labs, Inc., Plainview, N. Y.) for 6 hr at 20°C. It was assumed that liquid diketene was 24.4 M. After reaction, the solution was exhaustively dialyzed against multiple changes of distilled water for 24 hr at 20°C. Using aqueous $FeCl_3$ solution the number of acetoacetyl groups attached was measured spectrophotometrically (13). The molar extinction coefficient of the acetoacetyl group in the presence of $FeCl_3$ was 371 at 540 nm.

Acetoacetyl groups bound to residues other than amino groups were removed by treating preparations with 0.2 M $NaHCO_3$-Na_2CO_3 buffer at pH 9.5 for 18 hr at 20°C (13). Buffer and reactants were then removed by dialysis against distilled water (24 hr, 20°C). All acetoacetyl groups were removed by treating the acetoacetylated flagellins with a 10-fold molar excess of hydroxylamine (pH 7, 20°C, 24 hr) with respect to the number of acetoacetyl groups attached (13). Reactants were removed by dialysis.

Dinitrophenylation of Flagellin.—The extent of acetoacetylation of lysyl amino groups was determined by amino acid analysis of dinitrofluorobenzene (DNFB) reacted protein. Approximately 200 μg of acetoacetylated flagellin was dissolved in 100 μl of 0.1 M, $NaHCO_3$-Na_2CO_3 buffer, pH 9.5, and reacted with 5 μl of DNFB (190 mg/ml in acetone) for 1 hr at 37°C in the dark. An additional 5 μl of DNFB was then added and the mixture left to react for another hour. Under these conditions all the lysines and methyl lysines of flagellin were dinitrophenylated whereas acetoacetylated lysines (and methyl lysines) were not substituted. The dinitrophenylated protein was precipitated by the addition of 0.9 ml of 10% trichloroacetic acid, pelleted by centrifugation (3000 g, 10 min), and washed twice with 0.9 ml of 1 N HCl. The precipitate was then hydrolysed in the presence of 0.5 ml of 5.54 N HCl (constant boiling) at 110°C for 4 hr. For convenience and to avoid losses of material, the same tube was used for dinitrophenylation, centrifugation, and hydrolysis.

Hydrolysates were applied to a Technicon amino acid analyzer (Technicon Corp., Tarry-

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[2] *Abbreviations used in this paper:* DNFB, dinitrofluorobenzene; ΔF_{rel}, average free energy of binding; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; K_{rel}, relative antigenic activity; ΔK, intrinsic association constant; σ, heterogeneity index.

town, N. Y.)and their content of lysine and ϵ-N-methyl lysine determined by developing the column with a pH 5.28 buffer (11).

Immunodiffusion (Ouchterlony) Reactions.—Immunodiffusion was performed on microscope slides in 1% (w/v) agar containing phosphate-buffered saline (pH 7.0). Antigen (100 µg/ml) was placed in the peripheral wells and undiluted, rabbit anti-flagellin antiserum in the center well. Reaction was allowed to take place at room temperature for 24 hr. The slides were then washed three times with saline and once with distilled water to remove soluble protein. Finally, the slides were dried and stained with a 0.25% (w/v) solution of amido black in 7% (v/v) acetic acid.

Immobilization Inhibition Test.—This technique determined the capacity of flagellin and its acetoacetylated derivatives to inhibit the immobilization of flagellated bacteria by anti-flagellin antibodies. The test has been published in detail elsewhere (14).

Microprecipitin Inhibition Test.—This technique has been described in detail previously (14, 15). Briefly, the method measured the amount of flagellin or acetoacetylated flagellin required to inhibit the precipitation of 10 ng of ^{125}I-labeled flagellin by rabbit anti-flagellin antiserum.

Analysis of Inhibition Data.—The "relative antigenic activity" (K_{rel}) of flagellin and its acetoacetylated derivatives was determined as follows (16, 17):

$$K_{rel} = \frac{\text{Amount of flagellin (50\% inhibition).}}{\text{Amount of acetoacetylated flagellin (50\% inhibition).}}$$

K_{rel} should approximate the reduction in the intrinsic association constant (ΔK) between flagellin and its homologous antibody caused by acetoacetylation. However, the multivalent nature of flagellin prevents K_{rel} from being a completely accurate estimate of ΔK.

Based on the assumption that $K_{rel} \simeq \Delta K$ the reduction in the free energy of binding between antigen and antibody was determined as follows (18):

$$\Delta F_{rel} = RT \ln K_{rel},$$
$$= 1268 \log_{10} K_{rel},$$

if the antigen–antibody reaction reached equilibrium at 4°C (i.e., the microprecipitin inhibition reaction).

The "heterogeneity index" (σ) is a probability distribution function of the free energy of interaction between antigen and antibody (16, 17). The larger the value of σ the greater the heterogeneity of binding energies. Theoretically, with a σ value of 1.0, 84% of the antibody molecules have a free energy of interaction with antigen within ±600 cal/mole of the mean binding energy (16). Values for σ were determined by a curve-fitting method proposed by Pauling, Pressman, and Grossberg (16).

Antibody Estimations.—Estimations of antibody to flagellin were made in two ways: (a) by bacterial immobilization, where dilutions of sera were tested for their ability to immobilize a standard preparation of motile *S. derby* which has the same flagellar antigens (fg) as *S. adelaide*, but has different O antigens (10); (b) by a technique involving agglutination of sheep erythrocytes sensitized with polymerized flagellin using chromic chloride. This technique will be described in detail elsewhere.

Both antibody assays gave comparable antibody titers.

Statistical Methods.—Standard errors of the means and P values according to the Student's t test were calculated by IBM computer.

RESULTS

Acetoacetylation Reaction.—The over-all acetoacetylation reaction is presented in Fig. 1. Earlier workers (12, 13) demonstrated that diketene or aceto-

acetic anhydride rapidly reacts with the α-amino group and lysyl ε-amino groups of proteins. In addition, the hydroxyl groups of tyrosine, threonine, and serine can become acetoacetylated when high concentrations of diketene are used.

All the acetoacetyl groups attached to proteins can be readily removed at pH 7 by the addition of low concentrations of hydroxylamine. The acetoacetyl-

$$H_2C = C - CH_2$$
$$\qquad | \qquad |$$
$$\qquad O - C \quad \text{diketene}$$
$$\qquad \qquad \|$$
$$\qquad \qquad O$$

$$+$$

$$HO-R \qquad NH_2-R$$

$$\begin{array}{cc} O & O \\ \| & \| \\ H_3C-C-CH_2-C-O-R \end{array} \qquad \begin{array}{cc} O & O \\ \| & \| \\ H_3C-C-CH_2-C-NH-R \end{array}$$

$$HCO_3^-$$

$$NH_2OH$$

$$HO-R$$

$$HO-R \qquad NH_2-R$$

$$+$$

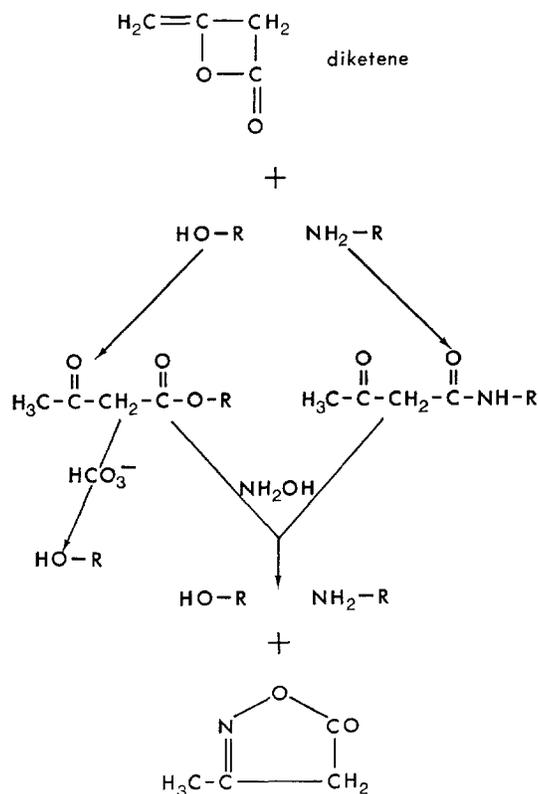$$\begin{array}{c} O \\ N \qquad CO \\ \| \qquad | \\ H_3C-C \text{———} CH_2 \end{array}$$

Fig. 1. The acetoacetylation reaction.

ated hydroxyl groups can be selectively unblocked by treatment with 0.2 M carbonate-bicarbonate (pH 9.5) solution (13).

Acetoacetylation of Flagellin.—Details of the reaction conditions are described in Materials and Methods. Flagellin (mol. wt. 40,000) from *S. adelaide* organisms contains 26 amino groups and 77 hydroxyl groups. The detailed composition of these groupings is presented in Table I. It should be noted that in addition to lysine, flagellin contains a substantial amount of the rare amino acid, ε-N-methyl lysine. Flagellin was reacted with molar excesses of diketene ranging from 19/1 to

960/1. The number of acetoacetyl groups attached to flagellin at each diketene concentration was estimated spectrophotometrically by measuring the absorbance of the protein at 540 nm in the presence of $FeCl_3$ solution. Fig. 2 depicts the results of these spectrophotometric measurements.

As the concentration of diketene was increased there was a steady rise in the number of acetoacetyl groups attached. Approximately 15 acetoacetyl groups were readily attached to the flagellin molecule, this level of substitution being achieved by a 96/1 molar ratio of diketene to flagellin. These 15 acetoacetyl groups were resistant to removal by bicarbonate solution and were therefore presumably attached to amino groups. Additional substitutions required much higher diketene concentrations, in fact, a 960/1 molar ratio produced

TABLE I

Content of Amino Acids in S. adelaide Flagellin Capable of Being Acetoacetylated

Amino acid	Residues/mole
Alanine*	1‡
Lysine	14‡
ε-N-methyl lysine	11‡
Tyrosine	6§
Threonine	36§
Serine	35§
Total	103

Data obtained from Parish and Ada (1969). The values are expressed as residues per mole, assuming the molecular weight of flagellin is 40,000.

* N-terminal amino acid of flagellin.
‡ Available $-NH_2$ groups = 26.
§ Available $-OH$ groups = 77.

only six additional substitutions (20.6 acetoacetyl groups/flagellin molecule). Furthermore, these additional substitutions were removed by bicarbonate, suggesting that they represented acetoacetylated hydroxyl groups (Fig. 2). In all cases approximately 90% of the acetoacetyl groups were removed by hydroxylamine solution (Fig. 2).

The extent of reaction of lysyl amino groups was also estimated by the amino acid analysis of DNFB-substituted flagellin (Fig. 3). Using this technique the extent of acetoacetylation of lysine and ε-N-methyl lysine residues was determined. Estimates of the number of acetoacetylated lysines at different diketene concentrations agree well with the spectrophotometric estimates of the number of substituted amino groups (15 substitutions/molecule). Thus, it appears that the α-amino group of flagellin is not being acetoacetylated, although more direct evidence is required to prove this point.

Amino acid analysis revealed that only 8/14 lysines and 7/11 ε-N-methyl

lysines in flagellin could readily be acetoacetylated (Fig. 3). The susceptible methyl lysines were slightly more amenable to acetoacetylation than the reactive lysines. For comparison Fig. 3 also contains a plot of the number of acetoacetylated OH groups at the different diketene concentrations, these values
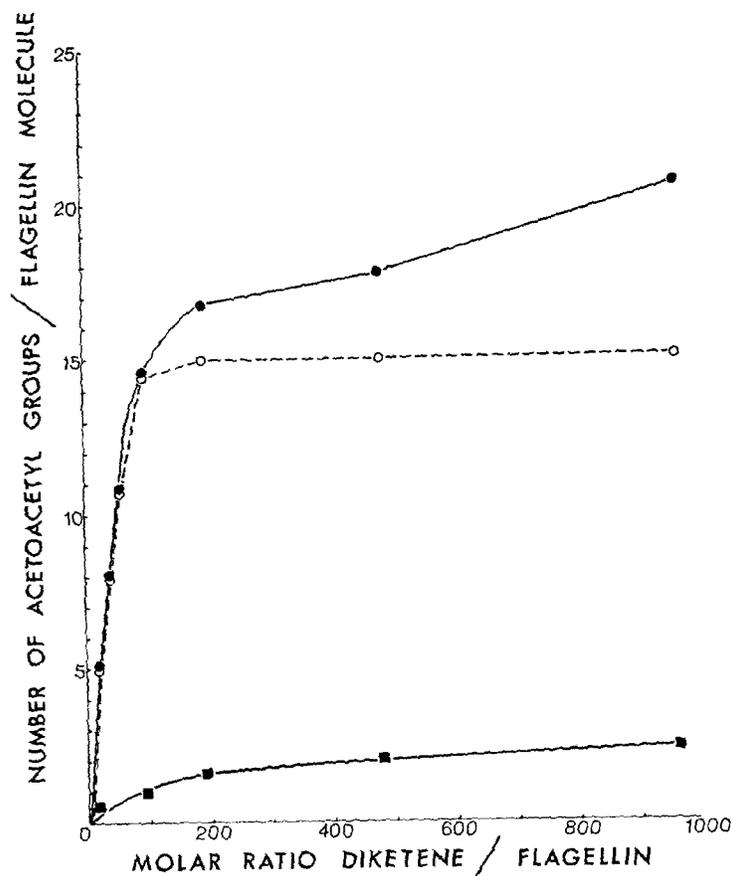

Fig. 2. Acetoacetylation of *S. adelaide* flagellin at pH 8.5 and 20°C. The extent of acetoacetylation was determined spectrophotometrically at 540 nm using 3% FeCl₃ solution. Total number of acetoacetyl groups attached (●——●), number of acetoacetyl groups attached after treatment with 0.2 M NaHCO₃-Na₂CO₃, pH 9.5 (○——○), number of acetoacetyl groups attached after NH₂OH treatment (■——■).

being obtained from the spectrophotometric data presented in Fig. 2. Again, it can be seen that all 15 reactive lysines in flagellin became acetoacetylated before any hydroxyl groups were substituted. The hydroxyl groups were very difficult to acetoacetylate, only 6 of the 77 hydroxyls in flagellin being substituted when a 960/1 molar ratio of diketene was used. It was not established

whether the acetoacetylated hydroxyls were derived from tyrosine, threonine, or serine residues. Substitution of the amino groups of flagellin was also monitored semiquantitatively by polyacrylamide gel electrophoresis at pH 2.7 in 9 M urea (11). With increasing amino group substitution there was a steady decline in the electrophoretic mobility of flagellin; in fact flagellin in which all 15 reactive lysines had been substituted did not enter the gels. No trace of unmodified flagellin could be detected in any of the preparations and all preparations which entered the gels formed narrow electrophoretic bands, an indication of highly homogeneous substitution.
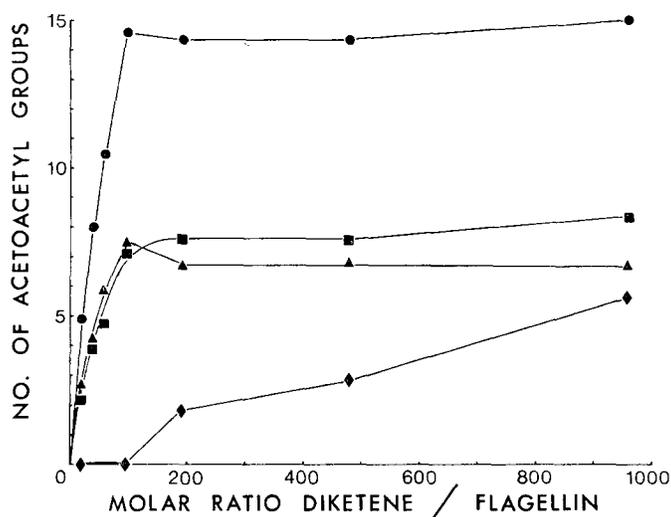

FIG. 3. Acetoacetylation of *S. adelaide* flagellin at pH 8.5 and 20°C. The extent o acetoacetylation was determined by amino acid analysis. Total number of acetoacetylated lysines and ε-N-methyl lysines (●——●), number of acetoacetylated lysines (■——■), number of acetoacetylated ε-N-methyl lysines (▲——▲), number of acetoacetylated hydroxyl groups (determined spectrophotometrically) (◆——◆).

Antigenic Properties of Acetoacetylated Flagellins.—The ability of the acetoacetylated flagellins to complex with anti-flagellin antibodies was determined by double immunodiffusion, microprecipitin inhibition tests, and immobilization inhibition tests.

Double immunodiffusion (Ouchterlony) test: This technique was a qualitative test of the ability of rabbit anti-flagellin antibodies to precipitate the acetoacetylated flagellins. Attachment to flagellin of up to 10.8 acetoacetyl groups resulted in no detectable loss in precipitating activity (Fig. 4 A). However, 14.6 acetoacetyl groups (96/1 preparation) produced faint spur formation. Additional substitutions enhanced spur formation and resulted in weaker and more diffuse precipitin lines (Fig. 4 B).

Microprecipitin inhibition test: Flagellin and its acetoacetylated derivatives were tested quantitatively for their ability to inhibit the precipitation of ^{125}I-labeled flagellin by rabbit anti-flagellin antibodies (14, 15).

Fig. 5 depicts the inhibition curves obtained with these antigens. In contrast to the immunodiffusion results, attachment of less than 14.6 acetoacetyl groups to flagellin did result in detectable losses in antigenic activity; in fact all the
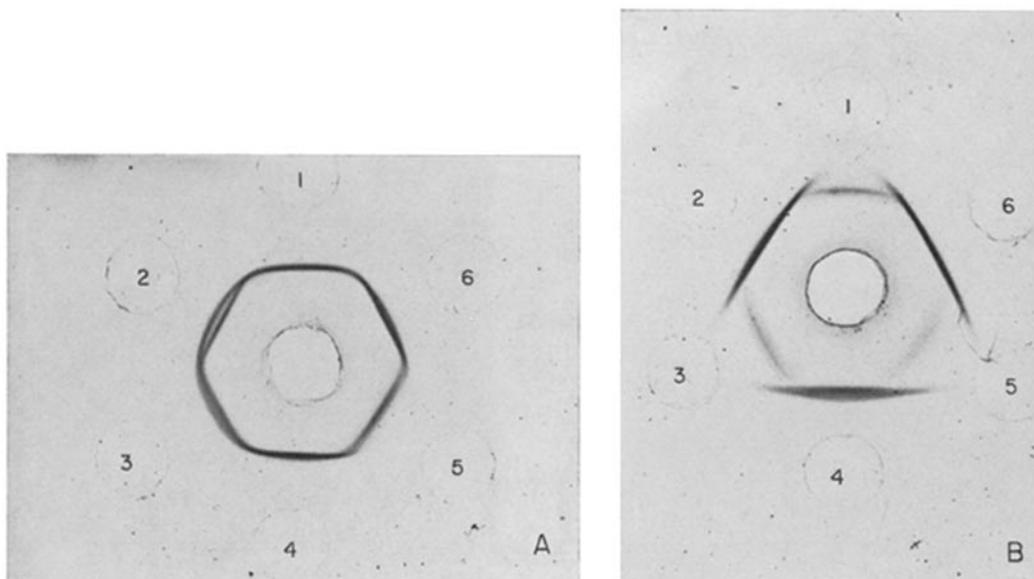

Fig. 4. Immunodiffusion reactions between the various acetoacetylated flagellins and rabbit anti-flagellin antiserum. The center wells contain the antiserum. (A) The peripheral wells contain: (1) flagellin—8.0 acetoacetyl groups; (2) unmodified flagellin; (3) flagellin—10.8 acetoacetyl groups; (4) unmodified flagellin; (5) flagellin—14.6 acetoacetyl groups; (6) unmodified flagellin. (B) The peripheral wells contain: (1) flagellin—16.8 acetoacetyl groups; (2) unmodified flagellin; (3) flagellin—17.8 acetoacetyl groups; (4) unmodified flagellin; (5) flagellin—20.6 acetoacetyl groups; (6) unmodified flagellin.

substituted flagellins exhibited a reduced capacity to bind with anti-flagellin antibodies. There was insufficient data to determine whether flagellin substituted with 16.8, 17.8, or 20.6 acetoacetyl groups could inhibit to completion (i.e., contained all the antigenic determinants of flagellin). Moreover, the 20.6 preparation produced no detectable inhibition at the concentrations used.

In Table II the inhibition curves are analyzed in detail. The amount of each antigen which produced 50% inhibition was determined (Table II, column 3). The K_{rel} of flagellin and its acetoacetylated derivatives was determined from the ratio of the 50% inhibition values (Table II). Approximate estimates of the

reduction in the average free energy of binding (ΔF_{rel}) between antigen and antibody were also obtained (Table II) (see Materials and Methods). The shapes of the inhibition curves (Fig. 5) enabled determinations of σ to be made, σ being an approximate index of the heterogeneity of the binding ener-
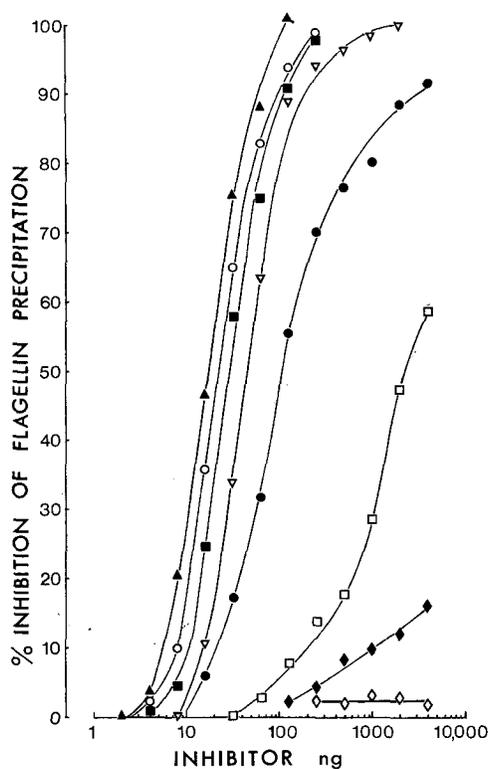

Fig. 5. The microprecipitin inhibition test. A comparison of the capacity of flagellin and the various acetoacetylated derivatives of flagellin to inhibit the precipitation of 10 ng of ^{125}I-labeled flagellin by rabbit anti-flagellin antibodies. The inhibition curves represent flagellin (▲——▲) and the following numbers of acetoacetyl substitutions: 5.0 (O——O); 8.0 (■——■); 10.8 (▽——▽); 14.6 (●——●); 16.8 (□——□); 17.8 (◆——◆); 20.6 (◇——◇).

gies between antigen and antibody (Table II). The larger the value for σ, the greater the heterogeneity.

As flagellin was acetoacetylated to increasing extents there was a steady decline in the affinity of flagellin for its homologous antibody (Table II). However, this reduction in affinity was *not* directly related to the number of acetoacetyl groups attached. Substitution with 5 acetoacetyl groups produced only a slight reduction in antigenicity ($\Delta F_{rel} = 185$ cal), whereas acetoacetylation

of all 15 reactive lysines (96/1 preparation) produced a much greater fall in antigenicity (ΔF_{rel} = 1040 cal) (Table II). The substitution of the reactive hydroxyl groups by higher concentrations of diketene (see earlier) resulted in very dramatic reductions in affinity. Substitution of 2.2 hydroxyl groups/mole increased the 50% inhibition value 22-fold, 3.2 hydroxyl substitutions 10,000-

TABLE II

Antigenic Activity of Flagellin and Its Acetoacetylated Derivatives as Measured by the Microprecipitin Inhibition Technique

Inhibitor	No. of acetoacetyl groups attached/ mole	Quantity of inhibitor for 50% inhibition*	Relative antigenic activity (K_{rel})	Hetero- geneity index σ	ΔF_{rel}‡
		ng			*cal*
Flagellin	0.0	17	1.0	0.25	0 (±300)
19/1§	5.0	24	7.1×10^{-1}	0.25	-185 (±300)
38/1	8.0	28	6.1×10^{-1}	0.25	-275 (±300)
CNBr digest of flagellin	0.0	30	5.7×10^{-1}	0.25	-312 (±300)
57/1	10.8	49	3.45×10^{-1}	0.25	-585 (±300)
96/1	14.6	112.5	1.5×10^{-1}	2.0	-1040 (±1200)
192/1‖	16.8	2500	6.8×10^{-3}	3.7	-2750 (±2200)
480/1‖	17.8	1.1×10^6	1.5×10^{-5}	?	-6100 ($\pm$?)
480/1-NH₂OH unblocked	2.0	36	4.7×10^{-1}	0.25	-418 (±300)
960/1‖	20.6	2.5×10^{10}	6.8×10^{-10}	?	-11700 ($\pm$?)

* Amount of inhibitor (ng) that gave 50% inhibition of precipitation of 10 ng of flagellin by rabbit anti-flagellin antiserum.

‡ Reduction in the average free energy of binding between antigen and antibody. Values in parenthesis represent the range in binding energy of 84% of the antibody molecules (callated from σ values). Free energy estimates are approximate rather than absolute (see Materials and Methods).

§ Molar ratio of diketene to flagellin.

‖ 50% inhibition values obtained by extrapolation (see text).

fold, and 6.0 hydroxyl substitutions 2×10^8-fold (Table II). Thus, it appears that the easily substituted lysine residues of flagellin are not intimately associated with the antigenic determinants of the molecule whereas the reactive hydroxyl groups play an important structural and/or antigenic role.

The heterogeneity index (σ) was unaltered by the attachment of up to 10.8 acetoacetyl groups/mole (Table II). However, more extensive substitution

rapidly increased the heterogeneity of binding energies. An important point to note is the remarkable homogeneity in the affinity of flagellin for the anti-flagellin antibodies. Presumably this is because the antiserum used constitutes a single bleed from one hyperimmune rabbit.

The 50% inhibition values for flagellin substituted with 17.8 and 20.6 acetoacetyl groups were not measured directly (see Fig. 5). However, it was found that there was a linear relationship between the molar ratio of diketene: flagellin and the $\log_{10}$ of the 50% inhibition values. This relationship was probably fortuitous but enabled an estimation of the 50% inhibition values of the 17.8 and 20.6 preparations to be obtained by extrapolation (Table II). These estimated values are consistent with the inhibition data in Fig. 5. Presumably the 17.8 and 20.6 preparations have high σ values (very heterogeneous binding energies).

Table II also summarizes the antigenic properties of CNBr-digested flagellin and acetoacetylated flagellin (480/1 preparation) which had been unblocked by hydroxylamine. CNBr digestion produced a slight reduction in antigenic activity, a fact which has been reported elsewhere (14). In terms of antigenic activity, a similar preparation of flagellin was obtained by the attachment of 8.0 acetoacetyl groups/mole. Removal of the acetoacetyl groups from flagellin by hydroxylamine restored the bulk of the antigenic activity of the molecule (Table II).

Immobilization inhibition test: The ability of the acetoacetylated flagellins to inhibit the immobilization of bacteria by anti-flagellin antibodies was determined, the results being presented in Table III. As was the case with the microprecipitin inhibition technique, acetoacetylation destroyed the antigenic activity of flagellin. However, the measured losses of antigenicity were approximately 2–2.5 times greater than those estimated by the microprecipitin technique. This was probably because in the immobilization technique (a) the antigen–antibody reaction was for a very short time (30 min) and therefore would not have reached equilibrium, (b) a subpopulation of antibody molecules may produce immobilization of bacteria, and (c) the immobilization technique was performed at 20°C (room temperature), whereas the microprecipitin test reached equilibrium at 4°C.

In accordance with the microprecipitin results, a CNBr digest of flagellin had similar antigenic activity to flagellin which had been substituted with 8 acetoacetyl groups (Table III).

In all the antigenic tests described so far, hyperimmune *rabbit* anti-flagellin antiserum was used. However, similar immobilization inhibition results were obtained when *rat* anti-flagellin antibodies were employed.

Antibody-Forming Capacity of the Acetoacetylated Flagellins.—Groups of adult

rats (seven per group) were injected into the hind footpads with 1 μg of one of the acetoacetylated flagellins in saline. Antibody titers were measured at weekly intervals and compared with those obtained in control animals which were injected with unmodified flagellin. Five wk later all animals were chal- lenged with 1 μg of unmodified flagellin in saline into the hind footpads and antibody titers determined up to 6 wk postchallenge. Figs. 6 and 7 present the results of this experiment. Four different preparations of acetoacetylated flagel- lin were studied which had the following antigenic activities relative to flagellin

TABLE III

Antigenic Activity of Flagellin and Its Acetoacetylated Derivatives as Measured by the Immobilization Inhibition Technique

Inhibitor	No. of aceto-acetyl groups attached/mole	Quantity of inhibitor for 80% immobilization*	Relative antigenic activity (K_{rel})	ΔF_{rel}‡
		ng		*cal*
Flagellin	0.0	55	1.0	—
19/1§	5.0	115	4.8×10^{-1}	−430
38/1	8.0	310	1.8×10^{-1}	−1000
CNBr digest of flagellin	0.0	275	2.0×10^{-1}	−940
57/1	10.8	625	9.1×10^{-2}	−1420
96/1	14.6	2200	2.5×10^{-2}	−2140
192/1	16.8	100,000	5.5×10^{-4}	−4360

* Amount of inhibitor (ng) that gave 80% inhibition of immobilization of motile *S. derby* (SW721) bacteria by rabbit anti-flagellin antiserum.

‡ Reduction in the free energy of binding between antigen and antibody. Free energy estimates are approximate rather than absolute (see Materials and Methods).

§ Molar ratio of diketene to flagellin.

(K_{rel} values): 6.1×10^{-1}, 4.7×10^{-1}, 3.45×10^{-1}, and 6.8×10^{-3} (see Table II).

Acetoacetylation readily destroyed the ability of flagellin to induce a pri- mary antibody response (Figs. 6 and 7). A 40% reduction in the antigenicity of flagellin (6.1×10^{-1} preparation) produced a 90–95% reduction in primary antibody formation. The 4.7×10^{-1} preparation produced only a trace of anti- body and the 3.45×10^{-1} and 6.8×10^{-3} derivatives produced no detectable primary response. Acetoacetylation also dramatically reduced the ability of flagellin to induce immunological memory (Figs. 6 and 7). Animals which had been primed and challenged with flagellin produced a mean secondary anti- body titer, 7 days postchallenge, of 3600. In contrast, rats primed with the

6.1 × 10⁻¹, 4.7 × 10⁻¹, and 3.45 × 10⁻¹ flagellins, when challenged with unmodified flagellin, produced antibody titers at 7 days postchallenge of 280, 76, and 16, respectively. No memory was induced by the 6.8 × 10⁻³ derivative. Control animals, which had received no priming before being challenged with flagellin, gave no detectable antibody 7 days postchallenge.
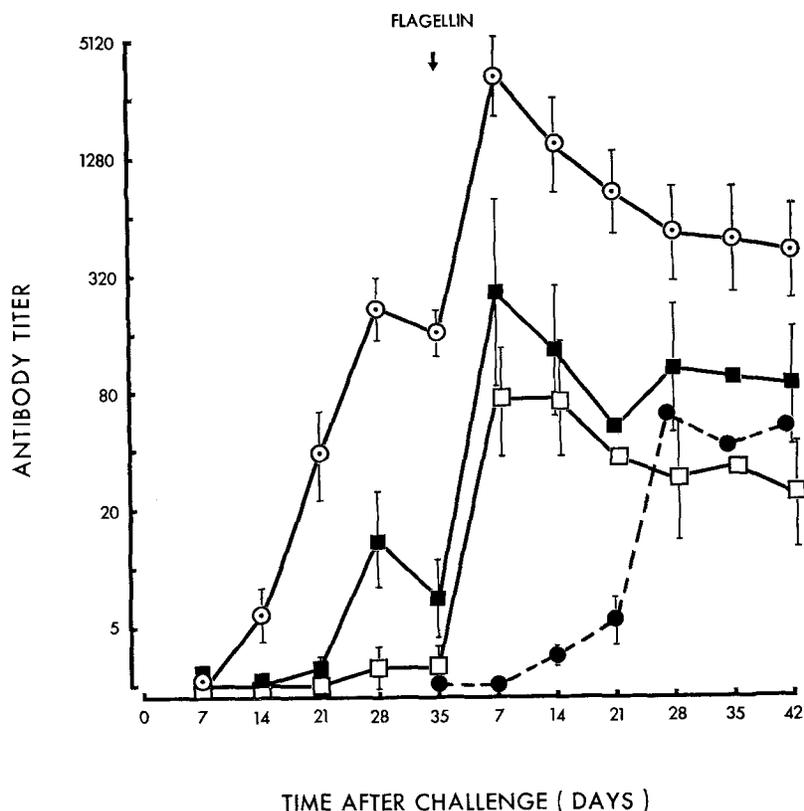
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FIG. 6. The ability of flagellin and its acetoacetylated derivatives to induce antibodies to flagellin (dose = 1 μg in saline). Rats challenged on day 35 with 1 μg of unmodified flagellin in saline. Legend: flagellin (○——○); flagellin—8.0 acetoacetyl groups, K_{rel} = 6.1 × 10⁻¹ (■——■); flagellin—hydroxylamine unblocked, K_{rel} = 4.7 × 10⁻¹ (□——□). Control rats (●---●) primed with saline and challenged on day 35 with flagellin (1 μg). Vertical bars are standard errors of the means.

Secondary antibody titers were measured for 6 wk postchallenge. Animsal which had been primed 5 wk previously with 1 μg of 6.8 × 10⁻³ flagellin had a very significantly reduced ability to respond to a subsequent challenge of unmodified flagellin (Fig. 7). Thus, by pretreating animals with the 6.8 × 10⁻³ preparation a state of partial immunological tolerance to flagellin was induced.

Suppression of antibody formation was of the order of 85–90% and this suppression persisted for the duration of the experiment. Although 3.45×10^{-1} flagellin induced slight immunological memory, the 28, 35, and 42 day post-challenge titers suggested that this preparation could also induce slight toler-
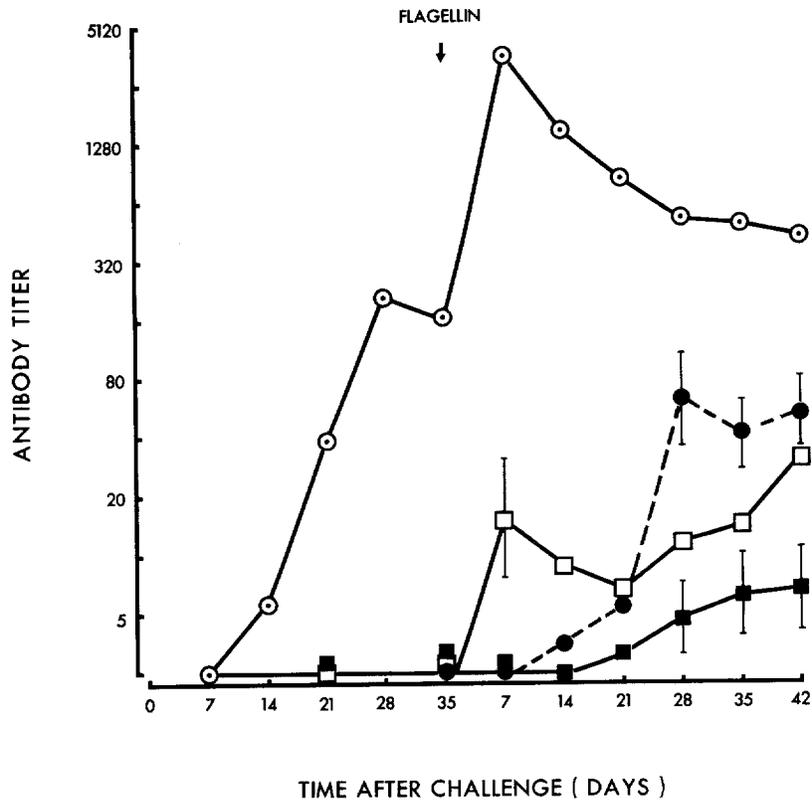
FIG. 7. The ability of flagellin and its acetoacetylated derivatives to induce antibodies to flagellin (dose = 1 μg in saline). Rats challenged on day 35 with 1 μg of unmodified flagellin in saline. Legend: flagellin ($\odot$——$\odot$); flagellin—10.8 acetoacetyl groups, $K_{rel} = 3.45 \times 10^{-1}$ ($\square$——$\square$); flagellin—16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$ ($\blacksquare$——$\blacksquare$). Control rats ($\bullet$---$\bullet$) primed with saline and challenged on day 35 with flagellin (1 μg). Vertical bars are standard errors of the means.

ance (Fig. 7). In contrast, the 6.1×10^{-1} and 4.7×10^{-1} flagellins produced substantial memory and not significant tolerance (Fig. 6).

Fig. 8 more clearly demonstrates the tolerance induced by the heavily aceto-acetylated flagellins. In this experiment animals were injected (hind footpads in saline) 5 wk previously with 1 μg of either 6.8×10^{-3} or 1.5×10^{-5} flagellin, and then challenged with 1 μg of unmodified flagellin in saline. Antibody

titers were measured up to 7 wk. postchallenge and compared with control animals which had been "primed" with saline. Both the 6.8×10^{-3} and 1.5×10^{-5} preparations induced highly significant tolerance ($P < 0.001$). However, the tolerance induced by these two preparations was not significantly different. In accordance with the experiment presented in Fig. 7, 6.8×10^{-3} flagellin produced approximately 90% suppression of antibody formation. Further-more, the antibody tolerance to flagellin appeared to be specific, rats pre-
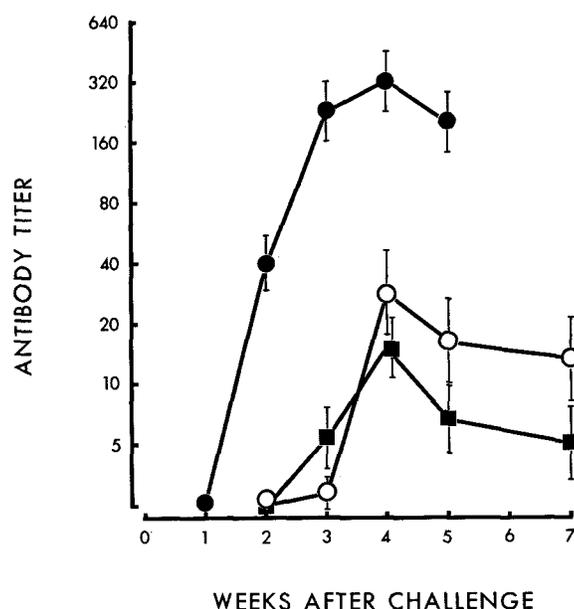

WEEKS AFTER CHALLENGE

Fig. 8. The "antibody tolerance" induced by heavily acetoacetylated derivatives of flagellin. Rats challenged 5 wk after priming with 1 μg of unmodified flagellin in saline. Legend: control rats—primed with saline, challenged with flagellin (●——●); primed with 1 μg flagellin—16.8 acetoacetyl groups, $K_{rel} = 6.8 \times 10^{-3}$ (○——○); primed with 1 μg flagellin—17.8 acetoacetyl groups, $K_{rel} = 1.5 \times 10^{-5}$ (■——■). Vertical bars are standard errors of the means.

treated with 1 μg of 6.8×10^{-3} flagellin producing a normal antibody response to an intraperitoneal injection of 1×10^8 sheep red blood cells.

Failure to Detect Antibodies Directed against Acetoacetyl Flagellin.—Sera from rats immunized with acetoacetyl flagellin were titrated against sheep red blood cells coated with the various acetoacetyl derivatives. In most cases no hemag-glutinating antibodies were detected. The exceptions were the less heavily acetoacetylated preparations which induced some hemagglutinating antibodies (see Fig. 6). However, these antibodies most effectively agglutinated red cells coated with flagellin, weakly agglutinated cells coated with mildly acetoacetyl-

ated flagellin (8.0 and 10.8 acetoacetyl groups/mole), and failed to agglutinate red cells tanned with the more heavily substituted preparations. It was demonstrated that the red cells were coated with acetoacetyl flagellin by using ^{125}I-labeled antigen. In addition no antibodies were detected which could precipitate 10 ng of ^{125}I-labeled acetoacetyl flagellin (16.8 acetoacetyl groups/mole). Under identical reaction conditions 10 ng of ^{125}I-flagellin was readily precipitated by rat anti-flagellin antibodies. The procedure was a modification of the microprecipitin technique (15).

DISCUSSION

It was demonstrated many years ago that extensive substitution of protein antigens by a wide range of reagents destroyed serological specificity (see reference 19). However, the ability of these substituted proteins to induce antibody formation has been frequently overlooked and their capacity to induce immunological tolerance to the unmodified protein has rarely been studied. Results presented in this paper demonstrate that acetoacetylation of the flagellin molecule also destroys antigenic activity. This loss in antigenic activity was accompanied by a fall in antibody-forming capacity and the conversion of flagellin into a molecule with strong tolerance-inducing properties.

Flagellin was readily acetoacetylated under mild conditions. These acetoacetylation studies confirmed the findings of other workers that the amino groups of proteins are much more easily acetoacetylated than the hydroxyl groups (12). However, only 15 of the 25 lysines in flagellin could be acetoacetylated, whereas earlier studies have demonstrated that all of the lysines in ribonuclease (12) and lysozyme (13) can be substituted.

Two types of antibody assays were used to study the antigenic properties of flagellin and its acetoacetylated derivatives. Firstly, the precipitin reaction was used either in a qualitative manner as simple immunodiffusion or as a quantitative microprecipitin inhibition reaction. Secondly, the technique of bacterial immobilization was used for inhibition studies. Several conclusions can be drawn from these antigenic tests.

(a) As flagellin was acetoacetylated to increasing extents there was a steady decline in its affinity for anti-flagellin antibodies. From the inhibition results estimates of the changes in the free energy of binding between antigen and antibody were determined.

(b) Antigenic activity was not related to the number of acetoacetyl groups attached, but was determined by the type of residue substituted. Reactive lysine residues were much less important antigenically than easily substituted hydroxyl groups.

(c) The heavily acetoacetylated flagellins had a very wide distribution of binding energies for anti-flagellin antibodies.

(d) The bulk of the antigenic activity of flagellin was restored when the acetoacetyl groups were removed by hydroxylamine.

It was difficult to determine from the antigenic tests whether extensive aceto-acetylation resulted in selective loss of antigenic determinants or in destruction of all determinants at a similar rate. Spur formation observed in the immunodiffusion reaction (Fig. 4 B) could be due to incomplete neutralization of *all* species of anti-flagellin antibodies by the acetoacetylated flagellins, rather than selective loss of determinants.

The immunogenicity studies demonstrated that acetoacetylation very readily destroyed the antibody-forming capacity of flagellin. However, this fall in immunogenicity was *not* related to the number of acetoacetyl groups attached but was determined by the antigenic activity of the preparations (i.e., the affinity of the acetoacetyl flagellins for anti-flagellin antibodies). This phenomenon was most clearly demonstrated by hydroxylamine un-blocked flagellin (Table II), which carried only 2 acetoacetyl groups/mole, but which had antigenic and immunogenic properties similar to flagellin that had been substituted with between 8 and 10.8 acetoacetyl groups (Fig. 6).

An important point to note is the dramatic change in the immunogenicity of flagellin following only minor changes in antigenic activity. The association constants for most antibody–hapten systems are about 10^5 M^{-1} (approximately 6000 cal) (20), although association constants as high as 10^{10} M^{-1} (approximately 13,000 cal) have been measured (21). Similar affinity constants have been obtained with protein antigens (20). Thus, it seems reasonable to assume that the flagellin–anti-flagellin reaction has an association constant somewhere within this range. However, a 40% reduction in the antigenicity of flagellin ($K_{rel} = 6.1 \times 10^{-1}$, $\Delta F_{rel} = -275$ cal) produced a 90–95% reduction in the antibody-forming capacity of the molecule. It can be concluded from this result that the affinity of antigen for the receptors on cells appears to be of crucial importance in determining whether antibody formation occurs. In contrast, immunological tolerance to flagellin was induced by acetoacetylated flagellins which had drastically reduced antigenicity (i.e., $K_{rel} = 1.5 \times 10^{-5}$, $\Delta F_{rel} = -6,100$ cal). One would expect these preparations to be unrecognizable as flagellin. However these derivatives have a very broad spectrum of binding energies, their immunological properties possibly being due to molecules with affinities close to that of flagellin.

There was no evidence that acetoacetylation converted flagellin into a new antigen which was capable of producing antibodies with a completely different spectrum of specificities. No antibodies could be detected which could precipitate heavily acetoacetylated flagellin or agglutinate sheep red blood cells coated with acetoacetylflagellin. Of course, these techniques would not detect small quantities of low affinity antibody.

It has been demonstrated previously in this laboratory that CNBr digestion destroys the antibody-forming capacity of flagellin but enhances the tolerance-inducing properties of the protein (8, 9). Now it appears that by a process of acetoacetylation even stronger tolerance-inducing preparations of flagellin

can be obtained. In fact, tolerance was only induced by multiple doses of the CNBr digest of flagellin (8, 9), whereas a single, 1 µg dose of acetoacetylated flagellin induces good suppression of antibody formation. Presumably the altered immunological properties of CNBr-digested flagellin are due to a slight loss of antigenic activity resulting from CNBr degradation (see Table II). Flagellin substituted with 8 acetoacetyl groups has similar antigenic properties to the CNBr digest. In the following paper (22) it will be demonstrated that these two preparations of flagellin do, in fact, have identical immunological properties.

It seems quite possible that acetoacetylation of many other protein antigens would also produce preparations with strong tolerance-inducing properties. In this paper no attempt has been made to interpret the results presented in terms of the inductive events required for antibody formation and immunological tolerance. These interpretations will be presented in detail in the following paper (22) where it will be demonstrated that acetoacetylation dramatically enhances the ability of flagellin to induce cell-mediated immunity.

SUMMARY

Flagellin (mol. wt. 40,000) from *S. adelaide* organisms was acetoacetylated to varying extents with diketene (acetoacetic anhydride). Chemical studies demonstrated that the amino groups of flagellin were more readily acetoacetylated than the hydroxyl groups. Several antigenic tests revealed that as flagellin was acetoacetylated to increasing extents there was a steady decline in the affinity of the molecule for anti-flagellin antibodies.

Loss in antigenic activity following acetoacetylation was not related to the number of acetoacetyl groups attached but was determined by the type of residue substituted. Reactive lysine residues were much less important antigenically than easily substituted hydroxyl groups.

Acetoacetylation very readily destroyed the antibody-forming capacity of flagellin in rats. This fall in immunogenicity was related to the antigenic activity of the preparations. In fact, only a 40% reduction in the antigenic activity of flagellin produced a 90–95% reduction in primary antibody formation. The more heavily acetoacetylated flagellins produced no detectable antibody and, in fact, rendered adult rats tolerant (in terms of antibody formation) to a subsequent challenge of flagellin. Tolerance was induced by acetoacetylated flagellins which had drastically reduced affinities for anti-flagellin antibodies.

These results were interpreted as indicating that the affinity of antigen for receptors on cells appears to be of crucial importance in determining whether antibody formation or immunological tolerance (antibody suppression) occurs.

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