compounds to undergo dehydration is also in accord with the hypothesis.

(4) *Selective dehydration of alcohols.* Dehydration of alcohols, such as $R_2CHCHOHCH_2R$, involving the possibility of loss of either an adjacent tertiary or an adjacent secondary hydrogen atom invariably proceeds by the first-mentioned path. Again, this is readily explicable in terms of the B-strain hypothesis.

Recent work has shown that the presence of two very bulky groups attached to a single atom may be the center of marked B-strain. The instability of such compounds as di-*t*-butylether and di-*t*-butylamine is ascribed to this cause. A quantitative estimate of the B-strain in such compounds has recently been made. Its magnitude, 5 to 7 kcal, is such as readily to account for the behavior of such substances.

It is believed that the B-strain hypothesis shows promise of being a fruitful unifying concept in organic chemistry. Experimental tests of the hypothesis are being prosecuted as vigorously as present conditions permit. It is hoped that these investigations will assist in drawing the attention of organic chemists to the desirability of including in their considerations both factors, polar and steric.

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The Detoxification by Acetylation of Soluble Antigens From *Shigella dysenteriae* (Shiga) and *E. typhosa*[1]

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The toxicity of available vaccines against typhoid and other gram-negative organisms has long been recognized as a serious defect, particularly under military conditions where the loss in personnel time due to vaccination reactions may be significant. Attempts to provide reasonable type-specific coverage against the more important dysentery organisms with polyvalent vaccines must also take into account the additive nature of the toxicities of the component organisms. The only solution at present is to reduce the amount of each component until the toxicity of the combination is within tolerable limits. Since the antigenic efficiency of a component is influenced by its concentration, it is obvious that additional injections must be made to reach a high level of protection, a procedure which apparently produces many administrative difficulties.

Numerous attempts have been made to detoxify either the whole organisms or selected antigenic fractions, but in spite of the application of a wide variety of reactions the loss in toxicity has almost invariably been accompanied by a corresponding decrease in antigenicity (*4, 6*). An exception may be noted for the action of ketene gas (*1*) on some gram-negative organisms, but this promising reaction does not seem to have been widely applied in practice.

In our studies on the development of a practical vaccine against *Shigella dysenteriae* (Shiga), we have favored the use of a soluble antigen—the somatic polysaccharide, or polysaccharide-protein complex (*6*). Although it was recognized that this might not be the only antigen responsible for protection, it was felt that elimination of less active components of the organisms might be advantageous, particularly if the material was to be incorporated into a polyvalent vaccine with the corresponding antigens of other members of the dysentery group. In searching for a method of detoxification we have noted the preparation by several workers of acetylated derivatives of bacterial polysaccharides (*3, 5*). Although quite complete chemical characterizations have been made, little or no data on the biological properties of the fractions are available, with the exception of the observation that acetylated antigens do not react well with antisera to the whole organisms.

We have therefore prepared a series of acetylated derivatives of the polysaccharide, or the polysaccharide-protein complex, of *Shigella dysenteriae* and of *E. typhosa* (Army strain 58) and have investigated their toxicity and antigenicity. The soluble toxic antigens were dissolved in a small amount of water, diluted with several volumes of pyridine, and allowed to react at room temperature with an excess of acetic anhydride. The general technique of Freeman (*3*) was followed, with the exception that samples were removed at shorter intervals, and the reactions stopped by pouring the aliquots into water. As acetylation proceeded, an increasing percentage of the antigens became insoluble in water.

In all cases even the water-soluble fraction isolated after the minimum time allowed for acetylation (1.5 hours) showed an appreciable decrease in toxicity over

[1] Preliminary report. The work described in this paper was done with the assistance of Miss Claire B. Abrams and Mr. Ben A. Rubin, under a contract recommended by the Committee on Medical Research of the Office of Scientific Research and Development, and Yale University. The experiments were begun under a similar contract with Harvard University.

that of the original material. The fractions water-insoluble after acetylation for 24 hours were the least toxic.

Toxicity was determined from the weight loss in mice. For a base line, six mice were given an intraperitoneal injection of 0.5 ml. of saline, which was the solvent or vehicle used for the other antigens, and the mouse weights determined daily. Other groups, of as near the same age and weight as possible, were given 0.5 ml. of antigen solution or suspension and the weights estimated in terms of percentage loss or gain from the control group. Four mice given 0.4 mg. of undetoxified Shiga soluble antigen all died; with 0.2 mg. ¾ died; with 0.1 mg. no deaths occurred, but there was a drop in weight which reached 13 per cent by the third day; with 0.05 mg. there was only a 6-per cent weight loss for three days.

At a greatly increased dosage (3.0 mg.) the 12 acetylated derivatives prepared from this antigen did not kill any of the 48 mice used. The weight losses of the injected mice varied from a maximum of 10 per cent for the less acetylated soluble fractions to zero for most of the water-insoluble fractions or more highly acetylated soluble fractions. By this method, then, the toxicity of many of the fractions was at least 60 times less than that of the original material.

Preliminary results with a small group of rabbits, which are especially sensitive to these toxins, indicate that a single injection of 0.005 mg. of the undetoxified Shiga antigen will produce a temperature rise of 2.7° C., with a characteristic initial leucopenia followed by a leucocytosis. On repeated injection, temperature rises as high as 3.6° C. have been obtained, with a leucocytosis of 36,000 after 18 hours. A sixty-fold larger dose (0.3 mg.) of the acetylated antigens produced responses which, for the less highly acetylated soluble antigens, were about equal to those given by 0.005 mg. of the toxic material, while the responses for the insoluble fractions were more moderate (temperature rises of 1.6° C., leucocyte counts of 12,000). The detoxification ratio of 60 or more for the most highly acetylated fractions was thus confirmed in these animals. It is possible that at least a part of the toxicity remaining in the soluble, less highly acetylated fractions is due to contamination with traces of the starting material, from which it is difficult to separate. The insoluble antigens, on the other hand, could be repeatedly washed free of soluble substances.

On intraperitoneal or subcutaneous injection into mice none of the acetylated fractions has yielded agglutinins for intact Shiga organisms, although these are readily produced by injection of the unacetylated, toxic complex. On first sight the fractions were thought not to be antigenic or, if so, of a specificity markedly different from that of the unacetylated material. That the latter is the case is indicated by the fact that more extensive immunization of rabbits has given rise to antibody which precipitates with acetylated fractions, although it does not agglutinate the intact organisms.

Of greater interest is the observation that mice given three subcutaneous or intraperitoneal injections of 0.05 mg. of the acetylated fractions do show active protection against infection with live Shiga organisms administered intracerebrally by Dubos' method (*2*). The data for three independent series of experiments are summarized in Table 1.

TABLE 1

SURVIVAL RATES OF IMMUNIZED MICE AFTER INTRACEREBRAL CHALLENGE WITH LIVE SHIGA DYSENTERY ORGANISMS

Immunizing antigen, given in 3 doses of 0.05 mg. each	Survivals	
	Number	Per cent
None—controls	4/55	7
Unacetylated, toxic Shiga antigen	4/15	27
Shiga antigens acetylated 1.5–4 hr.	19/93	20
Shiga antigens acetylated 5–24 hr.	64/151	42

It may be noted that the figures tabulated are for pools of animals; in some groups immunized with a particular fraction the survival rate was somewhat better (5/8), but the data for the smaller numbers of animals are not as significant statistically as are those for the combined groups. In a single experiment in which the challenge dose was administered intraperitoneally with mucin, 29 of the 61 mice (48 per cent) survived, as compared with none of 16 controls.

It should also be noted from Table 1 that the protection afforded appears to increase with acetylation, from which it may be concluded that the observed protection is not due to contamination of the acetylated antigens with small amounts of unchanged starting material. The protection in terms of multiples of MLD's is not impressive when compared to those of typhoid or Flexner dysentery protection experiments, but it is not out of line with that obtained typically in experimental Shiga infections.

Preliminary experiments with the protein-polysaccharide complex of *E. typhosa* indicate that significant detoxification of that antigen also may be obtained on acetylation. In a toxicity titration of the original soluble antigen in mice, the LD_{50} lay between 0.3 mg. (1/5 deaths, with 12 per cent weight loss) and 0.6 mg. (8/11 deaths). However, none of the 18 mice injected with 5.0 mg. of the acetylated fractions died, although some fractions produced transient weight losses of 10–15 per cent. The order of detoxification is thus about the same as for the Shiga antigen. The

protective power against active infection with typhoid organisms is now under investigation.

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On the Blocking Antibody and the Zone Phenomenon in Human Anti-Rh Sera

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Recent findings by Race (*7*), Wiener (*8*), and Diamond (*2*) indicate the importance of the indirect test as evidence of active isoimmunization in Rh– mothers of erythroblastotic infants. According to Levine (*6*), only 50 per cent of Rh– mothers who recently delivered infants suffering from this hemolytic disease have active anti-Rh agglutinins in their sera. In the remaining sera which fail to agglutinate Rh– blood, antibodies are present which specifically coat the surface of Rh+ red cells, rendering them resistant to the action of potent anti-Rh agglutinins. Diamond and Abelson (*3*) found that these so-called "incomplete," "blocking," or "inhibiting" antibodies will agglutinate heavy suspensions of fresh blood in tests carried out on slides. Presumably, the determining factor is the use of serum instead of saline as the medium for the cell suspension (*9*).

These significant findings on the behavior of blocking antibodies were confirmed. However, Diamond's observation that blocking sera will agglutinate heavy suspensions of washed blood could not be repeated. In order to facilitate studies on the specificity of the agglutination reactions given by blocking antibodies, Diamond's method was modified by the use of a 2-per cent cell suspension in human serum and test tubes instead of slides.

In the course of these confirmatory tests additional observations were made on the properties of those anti-Rh sera which exhibit the so-called zone phenomenon. Obviously, these sera contain both anti-Rh agglutinins and blocking antibodies, but the concentration of the former is greater. By and large, such sera were not considered as useful diagnostic reagents unless the agglutinin in a particular serum was far more active than the blocking antibody. However, the experiments presented indicate that at least some of these sera can be absorbed with Rh+ blood, so that after the blocking antibody is specifically removed the treated serum becomes a useful diagnostic reagent.[1] A typical experiment, given in Table 1, was carried out with the same serum originally described as exhibiting the zone phenomenon.[2]

TABLE 1

	Serum Sil.* Diluted 1:									
	2	4	8	16	32	64	128	256	512	1,024
Untreated	tr	±	+	+	+±	++	+±	+	±	0
Absorbed at 37° C.	+++	+++	+++	++±	++±	+±	+	±	0	0
Absorbed at 0° C.	+++	+++	++±	++	+±	+±	+	+	±	0

* The serum of patient Sil. was drawn in July 1942, and tests indicated in the table were carried out in July 1945. The undiluted serum was absorbed with an equal volume of washed sediment of Rh+ blood at both 37° C. and 0° C., but the agglutinin activity of the absorbed and unabsorbed sera were tested at 37° C. with 2-per cent saline suspension of Rh+ blood.

The test shows a somewhat higher titer with the serum absorbed at the lower temperature. Presumably, a slight quantity of the agglutinating activity was absorbed at 37° C., along with all of the blocking antibody.

Similar results were obtained with two other anti-Rh sera, one of which gave only doubtful reactions in the course of the titration. On absorption at 0° the two sera had agglutination titers of 1:32 and 1:16, respectively. The corresponding titers, after absorption at 37° C., were 1:2 and 1:4.

As was to be expected, the absorption mixtures with undiluted serum exhibited no gross agglutination; this was a favorable circumstance, because unagglutinated cells present a much larger surface area for specific absorption than agglutinated cells. This view could be confirmed in parallel absorption tests of pure anti-Rh agglutinins which require several treatments for complete absorption, whereas blocking antibodies of identical degree of activity were almost completely removed by one absorption.

The result presented in Table 1 could be duplicated in tests with a mixture consisting of equal parts of an anti-Rh_0 serum with agglutinating activity and another serum containing only blocking antibodies. On absorption of this mixture, which simulates to a remarkable degree the so-called zone phenomenon, it was possible to recover a great deal of the agglutinating activity (Table 2).

[1] After these experiments were completed, reference was found to similar absorption tests carried out by Wiener (*8*), but there is no indication that he tested the activity of the residual agglutinins by titration.

[2] The serum used in this test did not exhibit the zone phenomenon when first studied in July 1942. On standing for several months in the ice chest the serum was no longer found to be useful for routine testing because it exhibited the zone phenomenon to a striking degree (*5*).