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Preparation of an Artificial Antigen and Immunity to Mouse Typhoid

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Summary. A galactan isolated from gum arabic has been shown following O-acetylation to protect mice against *Salmonella typhimurium* infections. Data presented suggest that O-acetylated galactan induces in mice the formation of antibodies which cross-react with antigen 5 of *S. typhimurium*.

INTRODUCTION

Recent studies on *Salmonella typhimurium* infections in mice have demonstrated that specific antibody is a most important factor in immunity to this disease (Jenkin, Rowley and Auzins, 1964; Turner, Jenkin and Rowley, 1964; Rowley, Turner and Jenkin, 1964). Further investigations also showed that not all antibodies were equally effective in determining resistance, and preliminary results suggested that antibody against antigen 5 was crucial for the expression of immunity (Jenkin and Rowley, 1965).

In order to clarify this point, it seemed desirable to immunize mice with antigen 5 only, and to measure their immunity to subsequent challenge with *S. typhimurium*. The chemical structure of this antigenic site is relatively well known, and it would appear that the specificity of this antigen is determined by a terminal O-acetyl galactose (Kotelko, Staub and Tinelli, 1961). There seemed to be two possible approaches to this problem. One was to isolate the oligosaccharide unit (antigen 5) from the lipopolysaccharide of the intact bacteria and couple this to protein, forming a synthetic antigen, whilst the other was to prepare a synthetic antigen from a naturally occurring polysaccharide which would cross-react with antigen 5. The latter approach was chosen, and the work presented in this paper deals with the isolation of a galactan from gum arabic which on O-acetylation cross-reacts with antigen 5. The O-acetylated substance produces immunity in mice to *Salmonella typhimurium* infection.

MATERIAL AND METHODS

Preparation of galactan from gum arabic

The galactan was isolated from a commercial preparation of gum arabic (British Drug Houses, London) by the method given by Partridge and Morgan (1942). Galactan prepared by this method contained maximally 0·5 per cent protein as estimated by the method of Lowry, Rosebrough, Farr and Randall (1951).

O-acetylation of the galactan

The polysaccharide isolated above was O-acetylated by two methods which resulted in products containing from 2 to 20 per cent O-acetyl groups by weight.

Method 1: To 50 ml of acetic anhydride, 5 g of anhydrous sodium acetate was added. The mixture was heated over a steam bath for 5 minutes, and at the end of this period, 2 g of the galactan was added in small portions with constant agitation. Heating was then continued for a further 60 minutes, and the mixture was allowed to cool. Following cooling the contents of the flask were poured into 500 ml of ice-cold distilled water, and the product allowed to crystallize by standing overnight at 4° The acetylated polysaccharide was then filtered on a Buchner flask, washed several times with distilled water and finally dialysed overnight in the cold against distilled water. After dialysis the insoluble O-acetylated galactan was dried by lyophilization. This procedure results in insoluble polysaccharides containing from 2 to 8 per cent O-acetyl groups, the yield being 10–20 per cent of the theoretical. This method was also used for acetylating starch.

Method 2: This procedure was basically that described by Carson and Maclay (1946) for the acylation of polyuronides using formamide as a dispersing agent. To 5 ml of formamide at 50°, 500 mg of galactan was added in small quantities, and the mixture stirred at 45–50° for 60 minutes. Following the addition in small amounts of 4–5 ml of pyridine over a period of 30 minutes, the mixture was finally allowed to cool to room temperature. Three millilitres of acetic anhydride were added (1 ml every hour), and the reactants stirred for 5 hours. After standing overnight at room temperature, the mixture was poured into 100 ml of ice-cold 2 per cent hydrochloric acid, and the insoluble polysaccharide washed and dialysed as in 'Method 1'.

This method resulted in polysaccharides containing 20–25 per cent O-acetyl groups. The yield of O-acetyl galactan by this method was about 90 per cent of the theoretical.

The O-acetylated galactans were analysed for their O-acetyl content by the method given by Kabat and Mayer (1964).

Preparation of the protein component of the 'O' somatic antigen of Escherichia coli *K*12

The protein moiety of the 'O' somatic antigen of this strain of *E. coli* was isolated by the method of Partridge and Morgan (1942).

Conjugation of protein and O-acetyl galactan

The coupling of the protein component of the 'O' somatic antigen and O-acetyl galactan was achieved following the procedure outlined by Partridge and Morgan (1942). Only about 20 per cent of the O-acetyl galactan became conjugated under these conditions. The resulting complex contained 12 per cent protein and was not precipitable with trichloracetic acid. The O-acetyl galactan used in these experiments contained 2 per cent O-acetyl groups.

Determination of the molecular weight of the galactan

The molecular weight of $5{\cdot}5 \times 10^5$ for the galactan was determined by the sedimentation equilibrium method of Archibald (1947) in a Model E Spinco Ultracentrifuge and using the equations given by Schachman (1957). The Archibald run was carried out with a concentration of 9 mg/ml of galactan in 0·9 per cent saline, at a speed of 7928 rev/min, at a temperature of 20° and for 2½ hours. The synthetic boundary run (Co) was performed at a speed of 20,410 rev/min at 20° for 2 hours. The homogeneity of the polysaccharide

was determined in the ultracentrifuge at a speed of 59,780 rev/min at 20° using a concentration of 9 mg/ml of galactan in 0·9 per cent saline (Fig. 1).

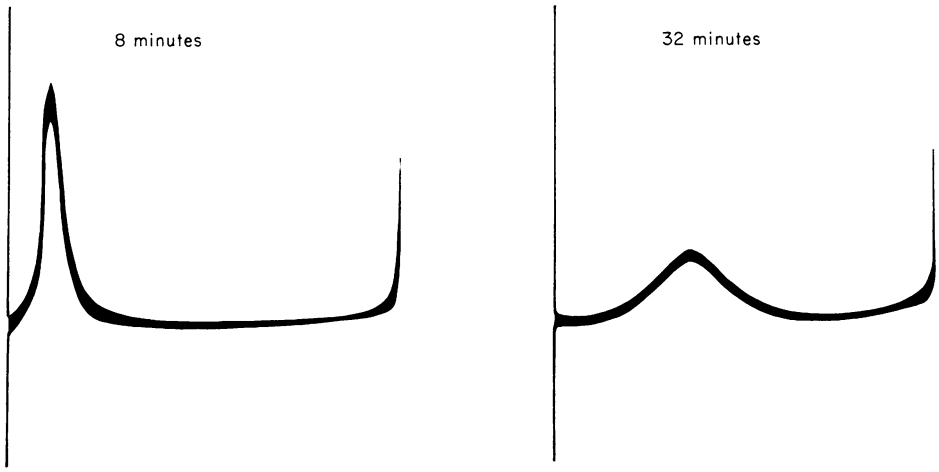

FIG. 1. Homogeneity of the galactan as indicated by the ultracentrifuge.

Bacterial strains

The strains of Salmonellae used in these studies were *S. enteritidis* 795 (Rowley *et al.*, 1964), *S. typhimurium* C5 (LD50 100–500), *S. typhimurium* H (LD50 500–1000) and an avirulent *S. typhimurium* (M206). In all experiments, unless otherwise stated, mice were challenged with approximately an LD100 dose of bacteria.

Immunization schedule

The insoluble polysaccharides were ground in a pestle and mortar. The resulting suspensions in saline were stable for several hours but settled out slowly on standing overnight. All potential antigens were given intravenously in 0·2 ml of saline, the various doses being indicated in the text. Mice were challenged with bacteria intraperitoneally 14 days after the immunizing dose and deaths recorded over a period of 28 days.

Strain of mice

This was a Swiss–Webster strain from Carworth Farms. All mice either male or female weighed 20–24 g at the time of immunization.

RESULTS

THE EFFECT OF IMMUNIZING MICE WITH VARIOUS ANTIGENS ON THEIR SUSCEPTIBILITY TO MOUSE TYPHOID

Mice were immunized intravenously with the following preparations; the doses injected are enclosed in brackets following the preparation.

Experiment 1

Protein–O-acetyl galactan conjugate containing 2 per cent O-acetyl groups (200 μg).

Thirty mice comprised the immunized group, and thirty mice injected with saline, the controls (Fig. 2).

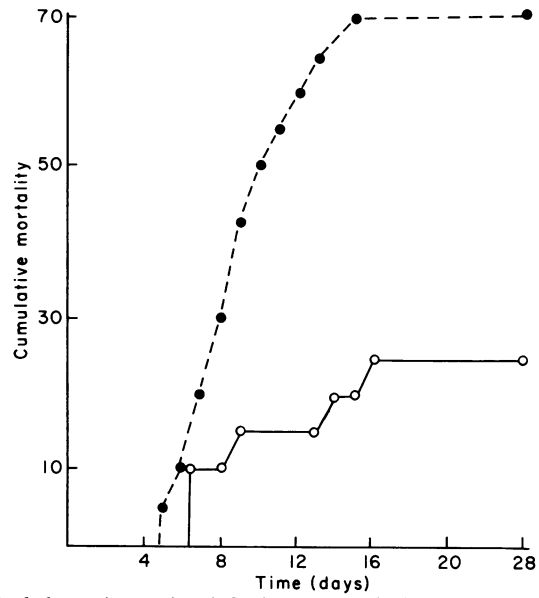

FIG. 2. Protection afforded to mice against infection with a virulent strain of *S. typhimurium* by immunization with 200 μg of a protein–O-acetyl galactan conjugate containing 2 per cent by weight O-acetyl groups. ●, Control group; ○, immunized group.

Experiment 2

Protein–O-acetyl galactan conjugate containing 2 per cent O-acetyl groups treated in the following manner: (a) heated at 100° for 60 minutes (200 μg); and (b) treated at a concentration of 1 mg/ml with 0·02 N NaOH for 20 minutes and finally brought to pH 7·0 with 0·02 N HCl (200 μg). In the above experiment twenty-five mice were included in each group (Fig. 3).

Experiment 3

O-acetyl galactan containing:

(a) 2 per cent O-acetyl groups
(b) 8 per cent O-acetyl groups } (50, 100, 200, 400 and 1000 μg)
(c) 20 per cent O-acetyl groups

each immunized group consisted of forty mice, while a total of sixty mice served as controls (Figs. 4 and 5).

Experiment 4

Galactan (200 and 1000 μg). The numbers of animals used in this experiment were similar to those in Experiment 1.

Experiment 5

Protein component of the 'O' somatic antigen of *E. coli* K12 (200 μg). Number of mice as in Experiment 1.

The data presented in Figs. 2 and 3 show that the protein–O-acetyl galactan conjugate

is capable of inducing an active immunity in mice to *S. typhimurium* infection. However, heat or alkali treatment of the complex destroys its immunogenic properties with reference to this infection (Fig. 3).

The results given in Fig. 4 comparing the immunizing efficacy of various O-acetyl galactans over a wide dose range are plotted as an index against micrograms of O-acetyl

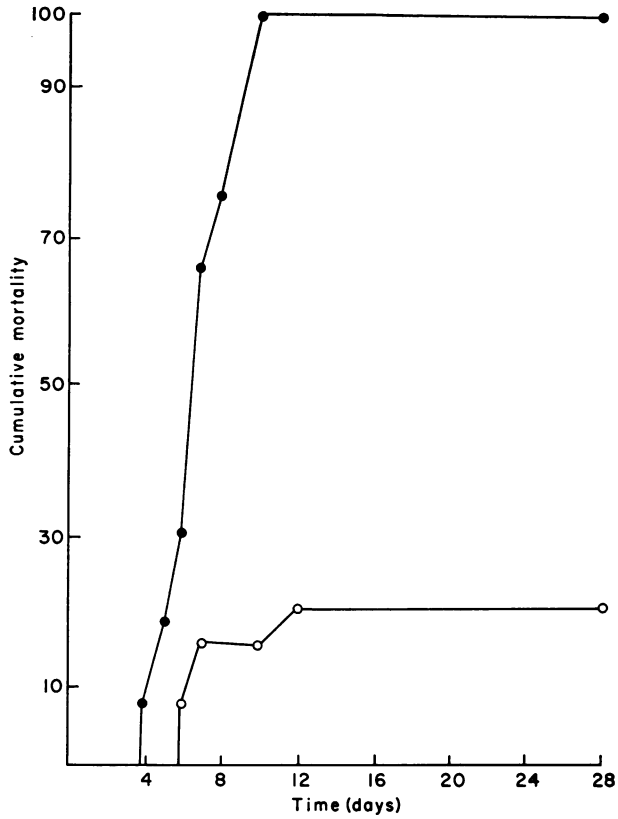

FIG. 3. Protection afforded to mice against infection with a virulent strain of *S. typhimurium* by immunization with 200 μg of a protein–O-acetyl galactan conjugate containing 2 per cent by weight O-acetyl groups that had either been heated or treated with 0·02 N sodium hydroxide. ●, Heat and NaOH treated antigen; ○, untreated antigen.

groups. The index has been determined in the following manner. Eighty per cent of the control animals had died at the end of 28 days, and this figure was given the value of 1·0. The survival index is given as:

$$1{\cdot}0 \times \frac{\text{Percentage mortality in control group}}{\text{Percentage mortality in immunized group}}.$$

Thus an index of 2·0 means that 40 per cent of the animals had died in that particular group given a certain dose of antigen. The values of the overall survival index were considered significant only when they were 1·33 or greater (Fig. 4). This method was found to be of value in comparing the course of the infection within the various groups, at different times, for it became apparent that certain low doses of O-acetyl galactan containing 8 and 20 per cent O-acetyl groups appeared to enhance the susceptibility of the mice

to the infection as measured by decreased survival time. Data measuring survival on the 8th day following challenge with bacteria and illustrating this point are shown in Fig. 5. At this time 28 per cent of the control animals had died.

It is clear from these results that mice may be protected against *S. typhimurium* infection by immunization with O-acetylated galactans whether or not they are conjugated to

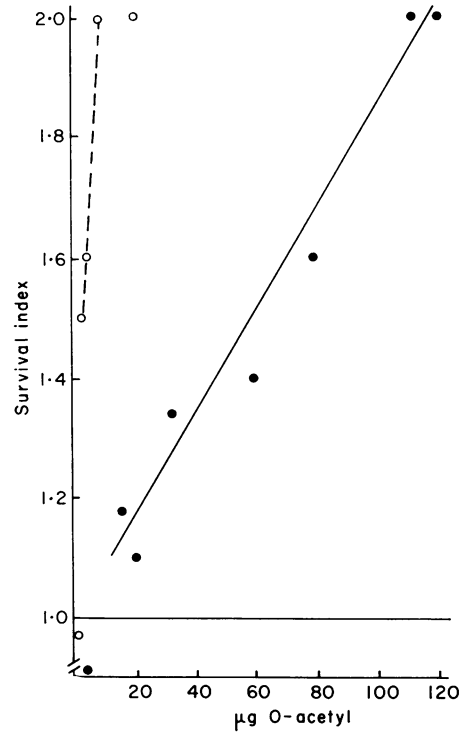

FIG. 4. The immunizing efficacy of various O-acetylated galactans over a wide dose range compared at 28 days. The survival index is determined by:

$$1{\cdot}0 \times \frac{\text{Percentage mortality in control group}}{\text{Percentage mortality in immunized group}}.$$

All mice challenged intraperitoneally with an LD100 of *S. typhimurium* H. ○, O-acetylated galactan containing 2 per cent by weight O-acetyl groups; ●, O-acetylated galactans containing 8 and 20 per cent by weight O-acetyl groups.

protein. The unacetylated galactan was found to be completely inactive as was the protein component of the conjugated protein–polysaccharide complex.

SPECIFICITY OF THE IMMUNE RESPONSE

Mice were immunized intravenously with 200 μg of O-acetyl galactan containing 2 per cent O-acetyl groups. Three days later these mice were given a second similar dose. The animals were divided into two groups and challenged intraperitoneally either with LD100 *S. typhimurium* C5 or with an LD50 dose of *S. enteritidis* 10 days after the last immunizing dose (Fig. 6). This experiment indicates that the immunity was specific for *S. typhimurium*.

INTRAPERITONEAL SURVIVAL OF *S. typhimurium* H IN NORMAL AND IMMUNIZED MICE

Mice were immunized intravenously with either 200 μg of O-acetyl galactan containing 2 per cent O-acetyl groups, with 200 μg of the same acetylated polysaccharide conjugated to protein or with 200 μg of galactan. Ten days after immunization, the mice were challenged intraperitoneally with approximately 10^4 *S. typhimurium* H in 0·2 ml of saline. At time 0 and at intervals thereafter over a period of 90 minutes, four mice were

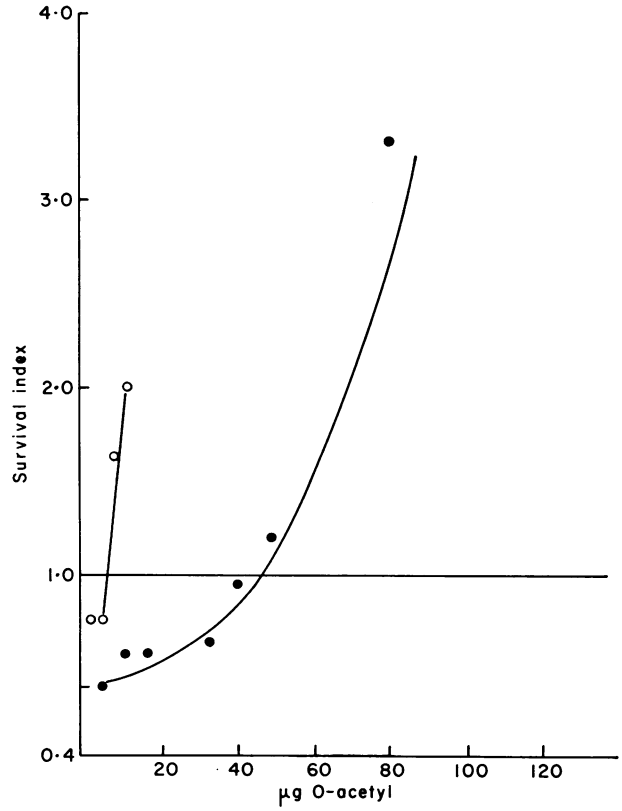

FIG. 5. The immunizing efficacy of various O-acetylated galactans over a wide dose range compared at 8 days. Survival index determined by:

$$1{\cdot}0 \times \frac{\text{Percentage mortality in control group}}{\text{Percentage mortality in immunized group}}.$$

All mice challenged intraperitoneally with an LD100 of *S. typhimurium H.* ○, O-acetylated galactan containing 2 per cent by weight O-acetyl groups; ●, O-acetylated galactans containing 8 and 20 per cent by weight O-acetyl groups.

killed and their peritoneal cavities washed out in the manner previously described (Jenkin and Rowley, 1965). An aliquot of the washings was immediately plated onto nutrient agar, and the bacterial population counted after overnight incubation at 37°. A group of non-immunized mice was similarly challenged, and the fate of the bacteria followed in a like manner. The results of this experiment are given in Fig. 7. No demonstrable killing intraperitoneally was noted in non-immunized mice or in mice immunized with the unacetylated galactan.

The experiment above was repeated with some modification. *Salmonella typhimurium* H

was opsonized with serum obtained from a sample of the immune mice above. The survival of the opsonized bacteria compared with unopsonised bacteria was followed in the peritoneal cavities of normal mice using the above technique. It was quite clear from the results obtained that the immunity of the mice, as measured by their capacity to kill *S. typhimurium* H in the former experiment, was dependent on the presence of antibody, since in this experiment a similar rapid rate of killing was obtained in the normal mice challenged with the opsonized bacteria.

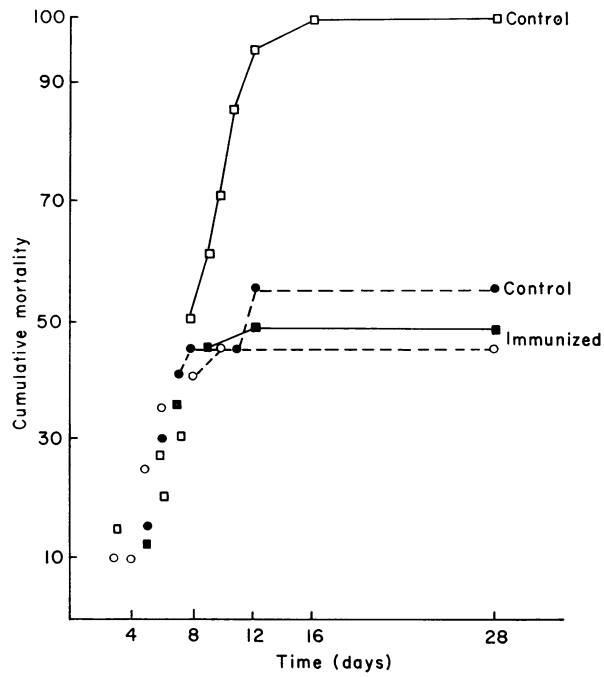

FIG. 6. The specificity of the immune response in mice injected with an O-acetylated galactan containing 2 per cent by weight O-acetyl groups and subsequently challenged intraperitoneally with either an LD100 dose of *S. typhimurium* C5 or an LD50 dose of *S. enteritidis* 795. There were thirty mice in each of the control and immunized groups. ○, Mice challenged with *S. enteritidis*; □, mice challenged with *S. typhimurium*.

FURTHER EXPERIMENTS ON THE CROSS-REACTION BETWEEN O-ACETYL GALACTAN AND *S. typhimurium*

The preceding experiments have shown that antibody produced in mice immunized with O-acetyl galactan cross-reacts with *S. typhimurium*. It was, therefore, of some interest to see if antibody produced in mice as a result of immunization with *S. typhimurium* cross-reacted with the O-acetyl galactan. Evidence for such a cross-reaction was obtained in the following manner. A large group of mice was immunized with the living attenuated strain of *S. typhimurium* M206 (Jenkin *et al.*, 1964). Ten days following immunization, the mice were divided at random into groups and treated in the following manner. Group (a) was bled from the retro-orbital plexus to provide immune serum. Group (b) was injected intraperitoneally with 4 mg of O-acetyl galactan (containing 2 per cent

O-acetyl groups). Thirty minutes after this injection, these animals were injected intraperitoneally with approximately 10^4 bacteria (C5), and the survival of the bacteria followed as previously described. Group (c) was injected with 4 mg of O-acetylated starch and then treated as for Group (b). Group (d) was injected with 4 mg of O-acetylated galactan as in Group (b) but was subsequently challenged with *S. typhimurium* (C5) that had been pre-treated (opsonized) with serum from Group (a). Group (e) was injected with 5 mg of galactan and then 30 minutes later treated in a similar manner to Group (b).

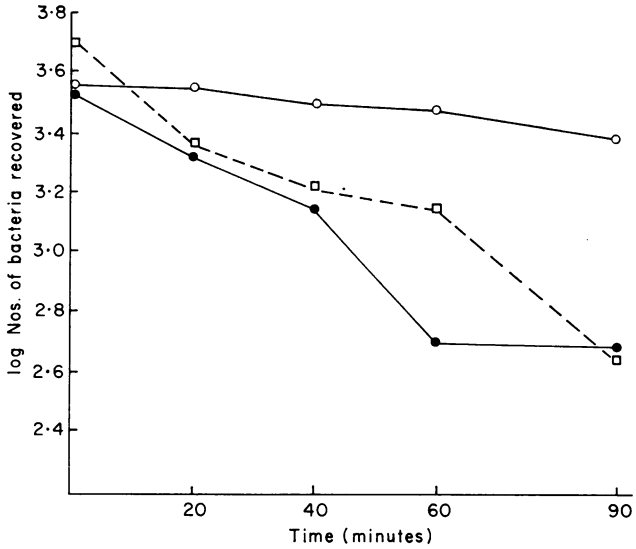

FIG. 7. Survival of *S. typhimurium* H in the peritoneal cavities of normal mice and mice immunized with various polysaccharides. ○, Galactan and normal control; □, protein–O-acetyl galactan; ●, O-acetyl galactan.

Group (f) was injected with 0·2 ml of saline and then 30 minutes later treated as for Group (b).

Normal mice served as controls to measure the survival of *S. typhimurium* under non-immune conditions. The results presented in Fig. 8 will be discussed later, but they show that the O-acetyl galactan competes with *S. typhimurium* for available antibody, as indicated by the inhibition of killing, in the O-acetylated galactan pretreated mice.

DISCUSSION

The results of this investigation show that galactans from gum arabic that have been O-acetylated are able to induce an immunity in mice against *S. typhimurium* infection. In general, there is correlation between the degree of immunity and O-acetyl content of the polysaccharide antigen. However, the polysaccharide containing 2 per cent O-acetyl groups by weight was a more efficient antigen than either of the other two preparations, namely those acetylated polysaccharides containing 8 per cent and 20 per cent O-acetyl groups. A tentative structure for the galactan has been proposed by Aspinall, Hirst and Nicolson (1959).

The polysaccharide is highly branched, the main chain consisting of 1–3 linked galactose residues. The side chains are attached by 1–6 linkages to each galactan in the main chain. Each short side chain consists of a galactose backbone terminating in an aldobiuronic acid. Further short chains are attached to these galactose residues and consist usually of either 3-O-L-arabopyranosyl-L-arabofuranosyl, 3-O-D-galactopyranosyl-L-arabofuranosyl, L-rhamnopyranosyl or L-arabofuranosyl, sugar units. However, it would

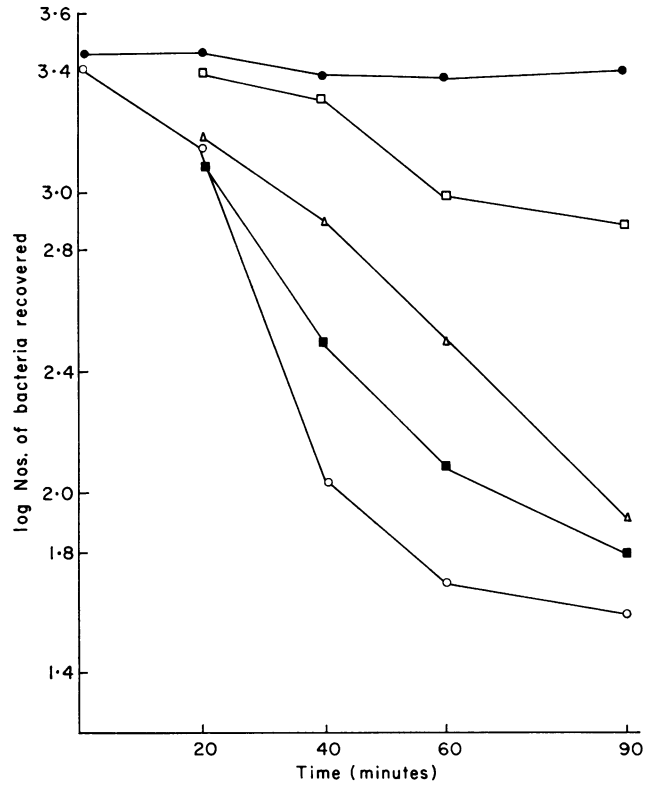

FIG. 8. Data illustrating the cross-reaction of antibody to *Salmonella typhimurium* with O-acetyl galactan containing 2 per cent by weight O-acetyl groups (see text for further details). ●, Control; □, O-acetyl galactan (Group b); △, Opsonized bacteria + O-acetyl galactan (Group d); ■, O-acetyl starch (Group c); ○, immunized mice (Groups e and f).

appear from work by Heidelberger, Adams and Dische (1956) that the galactan may consist of two structurally related polysaccharides which cannot be separated chemically but may be detected by immunochemical techniques.

The structure of antigen 5 would seem to be the folloing: O-acetyl galactose–mannose–rhamnose–galactose–mannose–rhamnose (Lüderitz, Staub and Westphal, 1966).

It is unlikely that the acetylated galactose units of the galactan resemble in any specific way the O-acetylated galactose sugar of antigen 5, though it is possible that this might by accident have been achieved in a small percentage of the galactose sugars. Nevertheless, it is apparent that not only does the antibody resulting from immunization with the O-acetylated galactan cross-react with *S. typhimurium* but that antibody against *S. typhimurium* reacts with the O-acetylated galactan. This latter aspect is indicated by the studies on the killing of *S. typhimurium* in the peritoneal cavities of mice that had been immunized

previously with an attenuated strain M206. The observed inhibition of the rate of killing after injection of O-acetylated galactan must be due to competition between the particles for the available antibody, since bacteria that were treated with immune serum prior to the injection were killed efficiently, and also the same weight of acetylated starch did not produce any striking inhibition of killing in these pre-immunized mice. These experiments indicate that it is the acetylated portion of the polysaccharide that is important in the inhibition, since galactan itself did not prevent killing at the dose level (5 mg) tested; yet excellent inhibition of killing was evident in the 2 per cent O-acetyl galactan pre-treated animals.

Immunochemical studies have shown that groups which are structurally related may cross-react, the intensity of the reaction depending on the degree of similarity between them. Thus antiserum to *S. typhi* cross-reacts with dextran, the cross-reaction appearing to involve antigen 12 (Staub, Tinelli, Lüderitz and Westphal, 1959), while anti-*S. paratyphi* B sera cross-react with certain galacto-mannans, the cross-reacting antigen being antigen 4 (Staub and Tinelli, 1957). This latter case may be a good example of the cross-reaction being dependent on similarity of structure rather than on identical structures (Kabat and Mayer, 1964). Evidence that the O-acetylated polysaccharide actually binds antibody was obtained from studies using the O-acetyl galactan in a column for the purification of anti-5 antibody (Jenkin and Rowley, personal observations). It has been shown with *Escherichia coli* 0·86, that antibodies against an antigen structurally related to an antigen of the bacteria are effective in promoting phagocytosis and killing (Pavillard, Stegemann and Rowley, 1964).

These, and the present observations, are important in our understanding of natural immunity and host defence mechanisms, since they imply that one specific antibody may serve as a recognition unit for a number of structurally related antigens.

Finally, the data presented in this paper support more conclusively the earlier observations that one requires antibody against antigen 5 to obtain good immunity in mice to *Salmonella typhimurium* infections.

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