Immunology of bovine heart valves

I. Cross-reaction with the C-polysaccharide of *Streptococcus pyogenes*

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The cross-reaction between the C-polysaccharide of *Streptococcus pyogenes* and material extracted from heart valves (Goldstein, Halpern, and Robert, 1967; Goldstein, Rebeyrotte, Parlebas, and Halpern, 1968) is potentially of great importance as an explanation of valvular damage in rheumatic fever. We therefore undertook a detailed examination of this system, but were unsuccessful in our attempts to confirm the existence of a cross-reaction. We also wish to record the chemical nature of the various valve fractions tested, and a number of possible technical snags which in our opinion may partially explain the previous results. In the second paper we describe the cross-reactions between heart valves and other connective tissues and draw attention to the potential usefulness of one of the valve extracts as a source of connective tissue antigens.

Materials

Armeen M2C was obtained from Armour Hess Chemicals Ltd, and Collagenase type III Fraction "A" (pfs) from Sigma London Chemical Company Ltd. Bovine heart valves were collected at a local slaughterhouse within 2 hrs

of killing the animals. They were frozen immediately with solid CO₂ and kept stored at -20°C. until required. The anti-streptococcal sera were those used by Kingston and Glynn (1971). They were raised in rabbits by intravenous inoculation of washed whole streptococci grown in a synthetic medium supplemented with tryptic digest of casein.

The properties of these sera are summarized in Table I. The rabbit antiserum to tubercle bacilli ("antiTB serum") was kindly supplied by Dr. E. Nassau of Harefield Hospital. It was prepared by injecting, intramuscularly in adjuvant and intravenously in saline, a phenol-killed washed culture of *Mycobacterium tuberculosis*, strain H37 Rv, grown in Dubos's medium. The sera were stored at -20°C.

BACTERIAL SUSPENSIONS FOR ABSORPTION
The *Streptococcus pyogenes* strains (see Table I) were grown in bulk at the Microbiological Research Establishment, Porton Down, in Oxoid tryptone soya broth and supplied as unkilld washed packed suspensions which were stored at -20°C. The Type 6 glossy suspension used for absorbing the antiTB serum was prepared by washing the thawed suspension twice with phosphate-buffered saline and re-suspending the packed cells in an equal volume of phosphate-buffered saline. The Type 6 matt

Table I Properties of antistreptococcal sera

Rabbit no.	4	10	13	14	15	16	18	19
Type of <i>Str. pyogenes</i>	24	6 matt	6 glossy	6 glossy	12	12	24	24
Group antibodies	±	+++	±	-	+++	-	-	-
Type antibodies	+++	+++	(+)	(+)	++	+++	+++	+++
Staining reactions								
Myocardium (sarcolemmal/subsarcolemmal)	+++	++	-	-	-	-	+++	±
Fibroblast	++	+	+++	-	-	+	++	±
Smooth muscle	++	++	++	-	-	++	++	±

- antibody not detected by the technique used.
± very weak reaction.
+ weak reaction.
++ strong reaction.
+++ very strong reaction.
The strains had the following numbers of the National Collection of Type Cultures: Type 6 glossy, 8709; Type 6 matt, 8302; Type 24, 8305. The Type 12 strain had the Streptococcal Reference Laboratory number R53/1077.
The presence of antistreptococcal antibodies was shown by reacting the sera with Lancefield and sometimes Fuller extracts (see Williams, 1958) of the homologous and heterologous strains in capillary tubes or by double diffusion in agar. The ability of the sera to give immunofluorescent staining of human tissues is condensed from Kingston and Glynn (1971).
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suspension used for absorbing the antiBHV sera was heat killed by suspending about 5 g. wet weight of cells in 10 ml. phosphate-buffered saline and holding the suspension at 56°C. for 1 hr. The killed suspension was passed several times through an X-press (A. B. Biox, Sweden) to break up the cells. The *M. tuberculosis* was kindly supplied by the Central Veterinary Laboratory, Weybridge, as a freeze-dried preparation. It was prepared from a mixture of strains of human *M. tuberculosis* which had been grown for 12 weeks on Dorset-Henley synthetic medium, killed by steaming for 3 hrs, and washed in distilled water. The suspension for absorption was prepared by suspending 0.174 g. in 2 ml. phosphate-buffered saline and treating it for 15 min. in a well-cooled M.S.E. ultrasonic disintegrator.

C-POLYSACCHARIDES FOR ABSORPTION

These were extracted with hot formamide from the bulk suspensions prepared for absorption (see above) with subsequent partial purification by repeated precipitation from acid alcohol with acetone (Erwa, Maxted, and Brighton, 1969). The strains used were Type 6 matt and A'. The latter was obtained from the Streptococcal Reference Laboratory, Central Public Health Laboratory (their reference 27A/32/3). A' (=A variant) strains carry a C-polysaccharide (group antigen) largely lacking N-acetyl glucosamine which is the determinant for the Group A antigen (see Krause, 1963). The C-polysaccharide prepared from the Type 6 matt contained 38 per cent. rhamnose, from the A' 63 per cent. These preparations were therefore probably less pure than those of Krause (1963) who gives figures of 60 and 85 per cent. respectively.

Methods

Chemical

The following standard techniques were used: Lowry, Rosebrough, Farr, and Randall (1951) for protein; Folin and Malmros (1929) for reducing sugars; carbazole method as described by Dische (1962a) for uronic acids; anthrone method for total hexoses (Yemm and Willis, 1954); Elson Morgan method as described by Dische (1962c) for hexosamines. Rhamnose was estimated by the cysteine or thioglycolic acid methods (Dische, 1962b). Hydroxyproline was estimated by the overnight procedure of Bergman and Loxley (1963).

PAPER CHROMATOGRAPHY OF SUGARS

The samples were hydrolysed in 0.5 N HCl for 18 hrs at 105°C. They were neutralized by shaking two or three times with 1 M chloroform solution of the organic base Armeen M2C. The water layer was separated each time by centrifugation (200 r.p.m. for 10 min.). The neutral solutions were passed a few times until ninhydrine negative through a small column (100 mg.) of Dowex 50 WX8 in its H⁺ form to remove amino acids (Partridge and Elsdon, 1961). The effluents were freeze-dried, dissolved in water, and applied to Whatman 1 filter paper. Ascending chromatography was performed in ethyl acetate:pyridine: water, 12:5:4, for 25 hrs and sugar spots were located by treatment with Ag NO₃ in acetone and Na OH in ethanol.

FRACTIONATION PROCEDURE (1) (Goldstein and others, 1967)

Bovine heart valves were thawed and washed with several changes of cold phosphate buffered saline, pH 7.2, to remove blood. They were homogenized in, and extracted at 4°C. overnight six or seven times with, CTC buffer. This buffer contained 1 M calcium chloride, 0.05 M Tris and 0.02 M citric acid. The final adjustment to pH 7.5 was made with citric acid. The buffer formed a precipitate when stored overnight, as could be expected from the concentration of calcium and citrate ions. This buffer was centrifuged and the supernatant, whose pH was unchanged, used for subsequent extractions. The extracts were separated by centrifugation at 2,700 r.p.m. in an MSE Major for 20 min. and the supernatants dialysed against water. The precipitate which formed was recovered by centrifugation, re-suspended in water, and freeze-dried. This material, using the nomenclature of Goldstein and others (1967), is referred to as crude soluble collagen (CSC). The supernatant which contained water-soluble material was also freeze-dried and is referred to as CTC extract. The insoluble residue left after extraction with CTC buffer was washed a few times with cold water to remove salts. It was then extracted three times with 3.8 per cent. trichloroacetic acid. Each time the suspension was heated up to 90°C. in a water bath, cooled in an iced water bath to 10°C. and centrifuged at the same temperature. The supernatants containing gelatin were discarded and the residue washed with a few changes of cold water to remove acid. The residue was then suspended in freshly prepared 8 M urea. Every extraction lasted 24 to 25 hrs. The extracts, which according to Goldstein and others (1967) contained valvular structural glycoprotein (VSGP), were recovered by centrifugation at 15,000 r.p.m. in a spinco ultracentrifuge (30 head) for 30 min. The part of it to be used for chemical estimation was dialysed against water and freeze-dried. The rest was dialysed against saline and kept for serological examination at 4°C. for immediate use or frozen at -20°C.

FRACTIONATION PROCEDURE (2) (Robert, 1969)

This was a modification of procedure (1) concerning the CTC buffer preparation and trichloroacetic acid extraction.

The CTC buffer was made fresh every day out of stock solutions of each component and used immediately before calcium citrate precipitation occurred. Trichloroacetic acid was used at a concentration of 2.7 per cent., instead of 3.8 per cent., and the extraction was carried out at 90°C. for 5, 20, and 30 min. in that order instead of simple heating up to 90°C. The fractions thus obtained by these more drastic methods are referred to as CTC-D, CSC-D, VSGP-D.

TRIAL EXTRACTION PROCEDURES

These procedures for isolating different glycoprotein fractions from heart valves were:

(1) Phenol extraction (Westphal and Jann, 1965; Procedure 1). A low yield of polysaccharide material was recovered from the aqueous phase.

(2) Mild NaOH extraction (Barnes and Partridge, 1968). This was used as an alternative to urea extraction after the homogenized tissue had been previously extracted with CTC buffer and trichloroacetic acid. The residue was

treated with 0.1 N NaOH for 30 min. at 4°C. with stirring, and the suspension neutralized and centrifuged. The material was recovered from the supernatant by dialysis and freeze-drying.

(3) Sodium deoxycholate extraction (Edgington, Glasscock, Watson, and Dixon, 1967). Homogenized bovine heart valves were extracted with 0.7 per cent. sodium deoxycholate and then precipitated with half-saturated ammonium sulphate or with ethanol.

Methods

Immunological

ANTISERA TO BOVINE HEART VALVES (BHV)

These were raised in rabbits using a homogenate of whole valves as recommended by Goldstein and others (1967), but with a modified schedule of injections. A fine tissue homogenate was made using an X-press (A. B. Biox, Sweden) or a cryostat. About 40 mg. dry weight of tissue was used for each injection. This was incorporated into Freund's complete adjuvant (Batco) for the first injection, and into Freund's incomplete adjuvant for the booster injection 3 weeks later. These injections were administered intradermally into various sites on the back. The rabbits subsequently received a series of subcutaneous injections of saline suspensions of heart valves spread over the following few months. Streptomycin and penicillin at 2 mg./ml. and 1,000 units/ml. respectively were incorporated to prevent infection of the injection sites. The rabbits were bled on the 8th day after the second injection and at the same interval after subsequent injections. The sera were stored frozen at -20°C.

QUALITATIVE PRECIPITATION

This was carried out either in capillary tubes or as single or double diffusion. In single diffusion the serum was incorporated into agar and left to solidify in the bottom of 2 mm. diameter tubes. The antigen solution was layered on the top, and moving bands of precipitate were observed when there was a positive reaction. Double diffusion was performed in plastic Petri dishes. 1 per cent. agar was made in phosphate buffer pH 7.2 and 0.1 per cent. sodium azide added as preservative. The thickness of the agar layer was 3 mm. The holes 3 and 5 mm. diameter were arranged in various patterns with distances between holes ranging from 7-10 mm. depending on the antigens and sera used.

QUANTITATIVE PRECIPITATION

This was performed as described by Kabat and Bezer (1945). Paired 0.5 ml. samples of antigen dissolved in phosphate-buffered saline at concentrations ranging from 125-2,000 µg./ml. were mixed with equal volumes of the sera tested. Controls omitting the antigen or serum were included. The tubes were incubated for 2 hrs at 37°C. and subsequently for 48 hrs at 4°C. The precipitates were spun at 4°C. for 30 min. at 2,500 r.p.m. in an MSE Major centrifuge and washed twice with chilled phosphate buffered saline. The proteins were dissolved in 0.1 N NaOH and estimated by Lowry's method.

PASSIVE HAEMAGGLUTINATION

The haemagglutination of formalized rabbit red cells to which antigens were coupled with the aid of bisdiazobenzidine (BDB) was done by the method of Butler (1963). The appropriate concentrations of antigens and BDB, and the percentage of normal rabbit serum in the diluent, were determined in trial experiments.

IMMUNOFLOUORESCENT STAINING OF STR. PYOGENES

Bacterial films were made with organisms grown in Oxoid tryptone soya broth. The cultures were washed once in phosphate-buffered saline and re-suspended in distilled water to an appropriate concentration determined by making a Gram film. Drops of the suspension were put onto ringed areas on cleaned microscope slides and allowed to dry. The films were usually fixed by heat as in making films for Gram-staining, but sometimes they were steeped in absolute ethanol for 15 min. The type 6 glossy was used except where otherwise stated as it showed a much smaller propensity for taking up immunoglobulins non-specifically than the other strains tested. The immunofluorescent staining was carried out substantially as described by Holborow and Johnson (1967), using a fluorescein conjugate prepared from a goat anti-rabbit γ-globulin (Hylands) and absorbed with human heart powder.

Results

The different fractions obtained by methods (1) and (2) were analysed. The results summarized in Table II show that CTC extracts prepared by either method were similar in their chemical composition and, as shown in the subsequent paper, immunologically similar. The VSGP-D, however, contained much less

Table II *Chemical analysis of valvular fractions*

Fractions	Hydroxyproline (%)	Hexoses as galactose (%)	Hexosamines as glucosamine (%)	Hexuronic acid as glucuronic (%)	Reducing sugars as galactose (%)
CTC	0.1-0.3	4.7-4.9	3.7-5.6	5.9	20.0
CTC-D	0.7	5.0		4.6	
CSC	2.0-2.4	3.1-3.8	2.1-2.5	3.7	11.5
CSC-D	6.6	3.9		2.8	
VSGP	4.0-8.0	3.6-4.2	1.3-1.7	1.3	11.7
VSGP-D	0.4	3.1		0.5	

The hexoses (identified by paper chromatography) in all fractions were: glucose, galactose, mannose.

collagen (estimated as hydroxyproline) than VSGP, correspondingly more being found in CSC-D.

Reaction of valvular fractions with antistreptococcal sera

Valvular structural glycoproteins and CTC fractions, which according to Goldstein and others (1967) contained the antigens cross-reacting with streptococcal Group A polysaccharide, were reacted with eight antiBHV (homologous) sera and with eight antistreptococcal sera in various immunological tests. Crude soluble collagen fractions were included in some tests.

In capillary tubes and in single diffusion, the valvular fractions gave good positive reactions with antiBHV sera. They gave no positive reactions with the antistreptococcal sera. The only exception was VSGP which gave a faint opalescence, but the preimmune sera gave a reaction of equal intensity.

In double diffusion none of the valvular fractions gave any specific reaction with the antistreptococcal sera. (Good reactions were given with the antiBHV sera; see subsequent paper.) CSC-D and VSGP fractions gave a faint line with all sera tested including preimmune sera. VSGP-D, which contained much less collagen than VSGP, did not give this line, nor did VSGP after treatment with the collagenase Type III (3 hrs, 37°C, 200 µg./ml. enzyme). The CSC-D had to be brought into solution by dissolving it in 8M urea and dialysing against buffered saline pH 7.2.

QUANTITATIVE PRECIPITATION WITH VSGP

Since weak precipitation was found when VSGP was reacted both with antistreptococcal and with the preimmune sera, a quantitative precipitation test was carried out to see if any significant difference could be found. Antistreptococcal serum 15 and six normal sera were examined. At an optimal concentration of 125 µg./ml. serum, the amount of antibody precipitated from serum 15 was 71 µg./ml., a figure which lies within the 16–91 µg./ml. given by the normal sera. When VSGP was reacted with antiBHV sera at the optimal concentration, the values varied from 372–415 µg. antibody per ml. serum.

PASSIVE HAEMAGGLUTINATION

Evidence of cross-reactivity was sought using the more sensitive technique of red cell agglutination. Formolized rabbit red cells were coated with CTC and valvular glycoprotein fractions using bis-diazobenzidine. These were used to titrate antiBHV, antistreptococcal, and preimmune sera. Cells coated with CTC extracts were agglutinated by antiBHV sera to a maximum dilution of 1/6400–1/12800. The corresponding titres for cells coated with VSGP and VSGP-D were 1/640–1/1280 for the former and 1/200–1/400 for the latter.

None of the antistreptococcal or preimmune sera gave any positive reaction with cells coated with any of the valvular fractions, at serum dilutions of 1/5 or above. The antistreptococcal sera tested were Nos. 10, 13, 14, 15, 16, 19.

REACTION OF FRACTIONS ISOLATED BY THE TRIAL EXTRACTION PROCEDURES

The three fractions, all at a concentration of 1 percent., were reacted with antiBHV sera and some antistreptococcal sera (10, 13, 15, 16) in capillary precipitation and double diffusion tests. Positive reactions were found only with antiBHV sera. Because of the absence of evidence for cross-reaction with streptococci, the fractions were not examined chemically, nor studied further immunologically except that the NaOH extract (procedure 2) was examined for its ability to inhibit macrophage migration (see subsequent paper).

Reaction of C-polysaccharide with antiBHV sera

This was examined by double diffusion in gel. The C-polysaccharide (crude) was prepared by Fuller's formamide method (see Williams, 1958) and at the dilution used contained 38 µg. rhamnose/ml. The antiBHV sera were taken from all eight rabbits. No lines of precipitation were found.

Ability of antiBHV sera to give immunofluorescent staining of *Str. pyogenes*

It was noticed that antiBHV sera stained *Str. pyogenes* Type 6 glossy to a variable extent. Sera withdrawn at intervals from two rabbits which received a prolonged course of immunization were examined. The staining was very weak with the earlier sera, and became definitely positive only later in the course of immunization. Since we could not confirm the existence of a cross-reaction between heart valves and the C-polysaccharide of *Str. pyogenes*, we looked for other explanations of the immunofluorescent staining of *Str. pyogenes*. It seemed possible that our results, namely a weakly positive reaction only after prolonged immunization, could be due to the slow development of antibodies to the cellular components of the valves, since these cells contain antigens which cross-react with streptococci (see Discussion). An additional explanation may be the presence of tubercle bacilli in the adjuvant used to raise the antiBHV sera, since these cross-react with streptococci (see below). As we used complete adjuvant for the first injection only, we feel that this explanation is probably more important for the results of Goldstein and others (1967).

CROSS-REACTIONS BETWEEN *Str. pyogenes* AND *M. tuberculosis*

All the sera used by Goldstein and others (1967) were raised with the use of Freund's complete adjuvant

and it was therefore important to see if there was a cross-reaction between *Str. pyogenes* and *M. tuberculosis* or *M. butyricum*. It was not possible to demonstrate cross-reactivity by double diffusion in agar gel using as sera: antistreptococcal sera 10 and 15, two antiBHV sera, and the antiTB serum; and as antigens: a Lancefield extract of *Str. pyogenes* Type 6M and Old Tuberculin (Evans Medical) at 1,000 units/ml. Because precipitation is not a very sensitive technique and because also relevant antigens might not be present in the bacterial preparations used, an immunofluorescent study was made.

It was found that films of *Str. pyogenes* Type 6 glossy were stained by antistreptococcal serum 15 and antiTB serum, the former very strongly, the latter strongly; that the two antiBHV sera gave weak and very weak staining respectively; and that a normal rabbit serum gave no staining. These sera were then absorbed twice with either sonicated *M. tuberculosis* at a concentration of 0.09 g./ml. serum or with whole washed cells of *Str. pyogenes* Type 6 glossy, equal volume of packed cells to serum. Unabsorbed sera had the appropriate volume of saline added and were treated in parallel (rotation for 30 min. on a haematology turntable at room temperature centrifugation, etc.). The results, summarized in Table III, show that antiTB sera react with *Str. pyogenes* and that this reaction is abolished by absorption with *M. tuberculosis*. The reaction with pooled antiBHV sera was not completely abolished by absorption with *M. tuberculosis*. A separate experiment showed that it could not be abolished by absorption with *M. butyricum* either.

Table III *Immunofluorescent staining of Str. pyogenes (Type 6 glossy) with antistreptococcal, antiBHV and antiTB sera. Effect of absorption with Type 6 glossy and M. tuberculosis*

Serum	Absorbed with		
	Nothing	TB	Type 6 glossy
Antistreptococcal 15	+++	+++	+
Pooled anti BHV	+	±	—
Anti TB	++	—	nd
Normal rabbit serum	—	—	—

nd: not done.

The antiTB serum and the antiBHV sera were absorbed with C-polysaccharide and the absorbed serum reacted with films of *Str. pyogenes* Type 6 glossy, to see if this antigen was responsible for the cross-reaction. If it were, it would provide a partial explanation of the results of Goldstein and others (1967). Fuller extracts of Type 6 matt and of an A' strain of *Str. pyogenes* (whose C-polysaccharide is largely deficient in the N-acetyl-glucosamine determinant) were compared to see if any effect found was

due to the C-polysaccharide or to other components present as impurities in the preparation. The anti-BHV serum was absorbed with 8 mg./ml. of C-polysaccharide, the antiTB serum with 4 mg./ml. Dilutions of the antiTB serum, with or without the C-polysaccharides, were applied to a film of Type 6 glossy. The results, recorded in Table IV, show that both C-polysaccharide preparations reduced the staining of *Str. pyogenes* to some extent, that from the Type 6 matt perhaps rather better. However, in view of the comparatively small difference in effect between the A and the A' C-polysaccharide, it was thought more likely that the effect was due to other cell constituents present as impurities in both.

Table IV *Effect of C-polysaccharides extracted from Str. pyogenes Type 6 matt and A' on the ability of antiTB serum to stain Str. pyogenes (Type 6 glossy)*

Absorbed with	Serum dilution		
	1/8	1/16	1/32
Nothing	++	++	+
C-polysaccharide 6 matt	+	±	—
C-polysaccharide A'	+	+	±

Discussion

The evidence adduced by Goldstein and others (1967) for a cross-reaction between the C-polysaccharide of *Str. pyogenes* and VSGP from bovine (and human) heart valves may be summarized:

- (1) Antistreptococcal sera gave lines in gel diffusion with VSGP, abolished by absorption of the sera with VSGP.
- (2) Antistreptococcal sera gave precipitates with urea extracts (VSGP) of bovine heart valves and CTC extracts of human heart valves.
- (3) AntiBHV sera precipitated C-polysaccharide extracted from group A streptococci, especially when this was linked to edestin.
- (4) AntiBHV sera gave immunofluorescent staining of streptococci of Group A but not of Groups C, G, and H, and this staining was abolished by absorption of the sera with the C-polysaccharide of Group A streptococci or with VSGP.

We were not able to confirm these results. Discussing the individual lines of evidence in detail:

- (1) We observed weak lines in the double diffusion test between VSGP and antistreptococcal sera, but we had to exclude them as being nonspecific: pre-immune sera gave the same lines. The lines were not given by VSGP-D. Our interpretation is that these lines were due to the collagen present in the former

fraction, as collagen is known to precipitate with serum proteins. This is supported by our finding that similar lines were given by the CSC-D fraction and that VSGP after treatment with collagenase no longer gave the lines. We cannot exactly compare our fractions with those of Goldstein and others (1967), since these authors give no information about chemical composition.

(2) There was no indication of any specific precipitation of VSGP with serum 15 (which had the strongest group activity) in the quantitative precipitation test. No positive results were obtained in the passive haemagglutination test when three valvular fractions (VSGP, VSGP-D, and CTC) were reacted with a variety of antistreptococcal sera, including sera 10 and 15 which had strong antigroup activity.

(3) None of our antiBHV sera reacted with our C-polysaccharide (prepared from the Group A Type 6 matt strain) in the gel diffusion test. We were not able to test the edestin complex.

(4) Our antiBHV sera only stained streptococci after prolonged immunization, although the sera taken earlier in the course of immunization strongly stained connective tissue components where structural glycoprotein antigens are most likely to be localized (see subsequent paper). Thus we could only find a very weak reaction in place of the strong one described by Goldstein and others (1967), and there are alternative serological reactions which we think explain many of these results.

The first is the cross-reaction between *Str. pyogenes* and *M. tuberculosis*. Goldstein and others (1967) raised their antiBHV sera using heroic amounts of complete adjuvant. (They also used some complete adjuvant for the anti-streptococcal sera.) A strong reaction between their antiBHV sera and streptococci is therefore to be expected. We only used complete adjuvant for the first injection, and our sera would therefore probably contain less antiTB antibodies. The ability of the antiTB serum to stain *Str. pyogenes* was partially abolished by C-polysaccharide preparations, and one prepared from the A' strain was very nearly as effective as that from Group A Type 6 matt. Thus the cross-reaction is apparently not due to the N-acetyl-glucosamine determinant, which is lacking in A' strains, and is probably due to impurities present in the formamide extracts. This is in keeping with the results of Karakawa, Lackland, and Krause (1966), who found that the immunological cross-reaction which occurred between bacterial mucopeptides of many species (mycobacteria however were not tested) was due to the peptide portion. We also feel that this shows that cross-reactions with formamide extracts must not be assumed to be due to the C-polysaccharide in them.

However, the cross-reaction between *M. tuberculosis* and *Str. pyogenes* may not completely explain the staining of streptococcal films with our antiBHV sera, as it was not completely abolished by absorption with sonicated *M. tuberculosis* or *M. butyricum*. It was found that sera taken after prolonged immunization stained cellular components present in heart valves, namely fibroblasts, endothelial cells, and myocardial fibres. These cells all share antigens with streptococci (Kaplan and Meyesian, 1962; Kingston and Glynn, 1971). Thus antiBHV sera will cross-react with streptococci owing to these cellular antigens. It is shown in the subsequent paper that absorption of antiBHV sera with VSGP or VSGP-D failed to abolish the cellular staining reactions of these sera. Thus at least some of the cellular antigens are not present in these fractions, which would probably not therefore react with streptococci.

Additional negative evidence comes from the comparative studies of the immunofluorescent staining of various tissues given by antiBHV and antistreptococcal sera (see subsequent paper). None of the antistreptococcal sera stained the non-cellular elements of connective tissue, though some gave strong staining of fibroblasts and endothelial cells. The antiBHV sera acted as general connective tissue reagents. After prolonged immunization, these sera also stained myocardium, fibroblasts, and endothelial cells, but we think that this was due to the presence of these cells in the valve homogenate. The failure of antistreptococcal sera to react with connective tissue in cryostat sections shows that any cross-reacting determinants in this tissue are unlikely to be exposed *in vivo*. It is possible that such determinants could be revealed by the chemical extraction procedure. Hidden determinants could not initiate an immunological reaction *in vivo*, though it is possible that they might perpetuate damage once started.

Apart from the two papers of Goldstein and others (1967, 1968), there are two other lines of evidence suggesting that there may be a cross-reaction between the connective tissue of heart valves and *Str. pyogenes*. Dudding and Ayoub (1968) found that antibodies to the C-polysaccharide of Group A streptococci remained elevated in chronic rheumatic valvular disease, but had an initial rapid fall in poststreptococcal diseases in which heart valves were not affected. If an antigen cross-reacting with the C-polysaccharide were present in the valves, it is plausible that the antibody would persist when there was chronic damage to the valves, either as cause or effect. However the flatness of the curve showing the die away of antibody in rheumatic valvulitis (their Fig. 7) is largely dependent on the initial point. We feel that this point is probably underestimated; the mean antibody level (measured by the Farr technique) corresponded to 87 per cent. precipitation of the radio-

actively labelled antigen. It seems likely that there was not sufficient antigen present to precipitate all the antibody in the higher titre sera. Their Fig. 10 shows that the antibody level to A-variant polysaccharide (which is formed by degradation of the C-polysaccharide) is also raised in rheumatic fever with valvulitis as against simple chorea. They state that the degree of elevation was not significant; however, that the antibody level was raised for all three points of the curve is itself significant. There is no cross-reaction claimed between heart valves and the A-variant polysaccharide, and since the polysaccharide is composed almost entirely of the bacterial sugar rhamnose it is most unlikely that there would be one. Thus we feel that the results of Dudding and Ayoub (1968) are suggestive of heightened immunological responsiveness, or possibly the presence of abnormal amounts of bacterial antigen, rather than the presence of a cross-reacting antigen.

The other line of evidence is that there have been other cross-reactions described between *Str. pyogenes* and human connective tissue components: glomerular basement membrane (Markowitz and Lange, 1964) and hyaluronate protein (Sandson Hamerman, Janis, and Rojkind, 1968). In view of the widespread sharing of antigens by different connective tissues, these seem to provide some support for the cross-reaction described by Goldstein and others (1967). However, in the system involving the kidney glomerulus, the cross-reacting antigen is located in the cytoplasmic membrane of the streptococcus, and is thus probably not the C-polysaccharide. Thus this system is unlikely to be the same

as that described by Goldstein and others (1967, 1968).

Summary

We attempted unsuccessfully to confirm the existence of the potentially important immunological cross-reaction between the C-polysaccharide of Group A streptococci and material extracted from bovine heart valves (BHV). Fractions were extracted from bovine heart valves with acid CaCl_2 solution (water-soluble and insoluble fractions), and from the residue (after treatment with trichloroacetic acid) with urea (Goldstein, Halpern, and Robert, 1967). The chemical composition of the fractions is given. They gave precipitates with antisera against whole valves but not with antistreptococcal sera, except that some fractions reacted weakly and non-specifically with all sera, immune and preimmune, probably owing to the collagen present. No reaction could be found between antistreptococcal sera and the BHV fractions coupled to red cells with bisdiazobenzidine. None of the antiBHV sera reacted with a streptococcal C-polysaccharide preparation in gel diffusion. It was however shown by immunofluorescence that antiTB sera would react with streptococci, and Goldstein and his co-workers used adjuvant containing tubercle bacilli in raising their antiBHV sera. Also the cells in heart valves (heart muscle fibres, fibroblasts) contain antigens shared with streptococci, and antibodies to these cells appeared after prolonged immunization with homogenized heart valve.

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Addendum

Since this paper was submitted for publication a further paper has appeared (Halpern, Parlebas, and Goldstein, 1971), in which a cross-reaction is claimed between the cell-sap of *Str. pyogenes* and anti BHV sera. We feel that this reaction might be explained by the *M. tuberculosis* in the adjuvant used to raise the antiBHV sera.