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The grouping of bloodstains falls naturally into two parts, the detection of antigens and the detection of antibodies. Dealing with the detection of the antibodies first I have little to say except that I consider it absolutely necessary before a bloodstain is reported on as being fully grouped in the ABO system that both antigens and antibodies should be proved.

THE DETECTION OF AGGLUTININS

The system we use at Harrogate is of the simplest, and merely entails placing small fragments of bloodstained fabric at the bottom of a small bore tube (about 5 x 0.5 cm) and dispensing onto it a small amount of 0.5% red cell suspension. The amount used is the smallest possible commensurate with recovering enough cell suspension to give a reading. In practice no extraction procedure can equal this simple process because quite apart from the extra manipulation required there are two factors mitigating against success in any system where extraction and reaction do not proceed simultaneously. The first of these is that as a bloodstain ages it becomes more insoluble and secondly, and this does not seem to have been explicitly recognised in the literature, that the dissolving of dried agglutinins in saline provides conditions where they can react with absorbing substances present in the fabric. It is quite obvious that should a fabric absorb strongly on the A side then a stain of Group O blood present on it will lose at least some of its anti A agglutinin. Drying of the bloodstain on the surface of the fabric will, however, frequently prevent this process going to completion, and this is shown in practice by some bloodstains showing an anti A on testing with red cells and yet showing A absorption. As far as I know this phenomenon has not been noted in the literature, but I have experienced it myself and I suspect that if extraction procedures were less popular then it would be noted as a common occurrence.

I also suspect that the reason one frequently fails to find agglutinins in bloodstains is because of absorption rather than deterioration but since one is subject to a continuous lecture on the lability of agglutinins in the literature of bloodstain grouping then the usual reason assumed for lack of success is destruction rather than absorption. It follows that if a bloodstain contains agglutinins in an absorbed condition then it should be theoretically possible to elute these from the fabric by raising the temperature. I have succeeded in doing this experimentally with clean cloth soaked in various quantities of saliva, but unfortunately in all the instances I have attempted to do this on actual case material then that old enemy of the bloodstain grouper—haemolysis—has intervened. If one could find a mode of treatment which would render red cells immune to haemolysis but still fully agglutinable then I would predict that the number of successes in agglutinin detection would go up sharply.

Both the absorption and the deterioration of agglutinins on bloodstained articles can be greatly reduced by correct conditions of storage. In our experience moisture causes more deterioration than temperature, and indeed it may be that a bloodstained object removed from a warm room to a refrigerator will suffer more reduction in agglutinin content than it would otherwise have done due to the concomitant increase in humidity when lowering the temperature. At the present time we favour the preservation of bloodstained articles by storage over P_2O_5 or $CaCl_2$.

A suitable technique for agglutinin detection is shown in Appendix A.

The Detection of Agglutinogens

The detection of antigens in bloodstains, at the moment, rests on the demonstration of the specific absorption of antibodies. Simple one step processes are not possible because of the destruction of the red cells by drying. Most of the problems of the bloodstain grouper would disappear if someone would discover a way of recovering cells from bloodstains in an agglutinable form. All the processes of liquid blood group serology would then be applicable to these cells.

We are then left with the necessity of carrying out absorption tests, and these tests fall into two classes, the first in which the absorption is demonstrated by the reduction of the agglutinating power of the antiserum. This is called the Absorption Inhibition process, a name which is in my opinion not a particularly good one since it suggests the inhibition of the action of an unchanged antiserum rather than the removal or neutralisation of an actual chemical entity. However it seems to be in such common usage that it would be difficult to change it.

The second method of demonstrating absorption is by proving not what is left in the antiserum but by proving what is combined with the bloodstain directly. This is the Absorption-Elution technique. Its main virtues are its simplicity compared with classical techniques and the fact that it takes only a fraction of the time of inhibition methods, is less prone to error and is one which the inexperienced worker can master quickly. It also proves actual absorption of antibodies which, strictly speaking, the Absorption-Inhibition test does not.

Absorption-Inhibition Tests. The basis of these tests is the ability of a bloodstain to reduce the agglutinating activity of an antiserum (or to 'inhibit' it). The simplest way to operate this test is to place a fragment of bloodstain in an aliquot of antiserum where it is left to absorb the agglutinins. The bloodstain is subsequently removed and known cells are added to the 'absorbed' antiserum. This process is described in Appendix B and in certain cases may be entirely satisfactory where, for example, the stain totally absorbs the activity of the antiserum and the control has no absorbing power at all. Unfortunately, in practice, straight-forward reactions are the exception rather than the rule and what frequently happens (apart from the fabric absorption) is that the bloodstain shows weak or no absorption and one is left wondering if this is a result of (a) the absence of the antigen tested for or (b) because the antigen is present in too small amounts or (c) because we have used an antiserum which is too strong. Absorption of agglutinins by the control is also, in practice, the rule rather than the exception especially where anti A reactions are concerned. I should mention here in passing that it is absolutely necessary that the control cloth used in bloodstain grouping should be taken from immediately adjacent to the stain under test. One still sees control taken from areas remote from the bloodstain under test and indeed when I was first shown how to group bloodstains I was told to take my controls from a remote area because (a) it nullified the chance of being contaminated with blood from the stain, (b) one could then choose an area which did not render the garment unusable and (c) one could pick a clean area in which the chance of interfering substances was remote!

If equivocal results are obtained from the simple procedure which I have just outlined then it becomes necessary to employ some form of titration. This is commonly carried out by making serial doubling dilutions of the absorbed and unabsorbed antiserum and comparing any change in titre. A better way of carrying out titrations in cases such as this is to use several dilutions of antiserum absorbed with constant amounts of stain either as an extract or in a powdered form. This system is more satisfactory but still time consuming. For a discussion of these techniques see Kind (1955). A method used in the Harrogate laboratories is outlined in Appendix C.

All these techniques are dependent on a number of variables which may nullify any results obtained or make necessary a redetermination. In order to make bloodstain grouping procedures more simple and accurate I embarked on a study which had as its initial objective the determination whether fresh cells could be attached to cells fixed on a slide and sensitised with an antiserum. I had no success with this (except occasionally with potent normal anti A sera) although it seems that Ogata (1960) has since then had some success with this method. The present technique is the result of this initial study, and it is put forward as a substitute for classical bloodstain grouping methods as being more efficient. It is not advocated as a panacea; but we have made several hundred determinations by this method, and we are con-

vinced that unit amount of information can be extracted from a stain in about a fifth of the time taken by absorption inhibition methods.

Absorption Elution Tests. We have tried several modifications of this test both in tubes and on slides. A simple demonstration of the method may be made as follows.

Fresh blood is diluted with approximately ten times its own volume of distilled water and approximately 50 μlitre lots are dispensed into the wells of cavity slides. This should be done for all the blood groups in the ABO system, and it is convenient to use for this purpose twin-cavity slides using one slide for each blood to be grouped. This enables the grouping to be conveniently carried out in duplicate. It is next necessary to fix this bloodstain onto the slide by such a method that will render the bloodstain insoluble and yet have no effect on the agglutinogens. ABO agglutinogens are thermostable to boiling water, and so we fix merely by plunging the dried slides into boiling McIlvaine buffer for 30 secs. at pH 7.4. It may possibly be that it would be equally satisfactory to use distilled water for the fixation, but we use buffer since in practice one is liable to meet with acid or alkaline substances much more than in the antiseptic precints of a blood grouping laboratory. It may be that there is some method of fixing other than boiling which would be equally satisfactory, but we have had no opportunity of experimenting in this direction. If the method is extended out of the ABO system, then obviously some other method of fixation must be found, but all I have to say here refers to tests made in the ABO system.

The slides are next sensitised with the necessary antisera and allowed to absorb for a period of two to three hours. Since the reaction of ABO antibodies and antigens is reversible and the equilibrium lies further towards the combined side at lower temperatures then we find it advantageous to carry out the final 30 minutes of absorption at about 4°C. This, however, is not absolutely necessary, and good results are usually obtained without it. The well of the slide is in each case filled with antiserum. The amount used is not critical, and the only criterion to observe is that excess should be used so that antigen is saturated with antibody under the conditions of the test.

Next, after the period of absorption the antiserum is washed off the slides with cold saline for a period of at least a minute. The slides are then quickly blotted dry. This process should be carried out as quickly as possible since elevation of the temperature of the slide from refrigerator temperature to room temperature will have some eluting action. Next the indicator cells are pipetted into the cavities of the slides which are removed to moist chambers at 50° to 55° Centigrade for ten minutes (the antisera used must, of course, contain no haemolysins). Next the slides are removed to room temperature where they are read after five and fifteen minutes. The concentration of indicator cells used is higher than that used in liquid bloodstain grouping since we find it only necessary to read the results with the naked eye.

It is probably better to err on the side of too concentrated a cell suspension rather than too dilute since the process is self-compensating and the higher the cell concentration the more competitive removal of agglutinin from the stain occurs. (Taking 60 μl of 3% cells and assuming that 45% cells contain 5,000,000 cells per μl then this volume of 3% cells will contain 20,000,000 cells each of an average surface area of 0.00013 mm^2 which will give a total surface area of 26 cm^2.)

The actual size of a blood smear grouped by this method is about 1 cm^2. The actual surface area will be somewhat greater than this due to microscopic irregularities. On the other hand some of the receptor groups on the surface of the bloodstain will be occluded by denatured serum proteins. Diffusion of antibody molecules may occur over a short distance through this denatured protein, but it is safe to say that the effective area of the bloodstain is certainly smaller than the surface of the cells onto which the antibody is being eluted. It would be interesting to elute for a second time with a fresh batch of cells without re-absorbing and see how much antibody comes off. I would suspect that the amount would be very small since convection currents caused on eluting by heating the slide from room temperature to 50°C would carry the antibody molecules into the body of the liquid and disperse them where they would be preferentially absorbed by the indicator cells. One must not be led into using very dilute cells in the hope that thereby the technique will be rendered more sensitive. Although this may work quite well in experimental smears, in actual case examples of bloodstain grouping the fabric nearly always causes some interfering absorption on the control side and it may be that the small amount of antibody eluted from this will be enough to agglutinate a small amount of dilute cell suspension.

Bloodstains on glass, metal, or similar impermeable surfaces are best treated in this way by scraping into the cavities of a cavity slide. Distilled water is added to the powdered blood, mixed, and allowed to dry. After fixation the procedure is as outlined above. Should the bloodstains be on fabric and difficult to remove the procedure adopted should be that described by Kind (1960 b).

The Barrier to Antibody Molecules

On fixing a bloodstain to be grouped by the elution method a surface of insoluble proteins is made in which are occluded the blood group substances. This layer of insoluble protein provides a barrier which is substantially impermeable to blood group agglutinins, a fact which is demonstrated by the following observations.

In day-to-day bloodstain grouping it is commonplace to encounter fabrics containing blood group activity not associated with blood. This is especially so in relation to blood group A activity, and often a garment will show anti A absorbing properties which are not nullified by the fixation process, that is, they are water insoluble.

If a stain of group O blood is made on such a fabric and fixed in situ by boiling, then it can usually be demonstrated that the anti A absorbing power of the fabric is nullified in the area of the bloodstain thus proving that the bloodstain presents an impermeable barrier to the agglutinin molecules.

A related observation we have made is that smears prepared from whole blood diluted with isotonic saline absorbs homologous agglutinin less strongly than smears which are prepared from the same blood diluted with water (and are thus haemolyzed). This observation has been made in about a dozen different experiments and is fairly consistent although the difference is sometimes marginal.

We have also carried out some preliminary experiments on the trypsinising of fixed blood smears and have found, contrary to expectations, that short term trypsinising (2 mins. at 15°C) reduces the absorbing power of the blood smear for homologous agglutinins compared with the untreated control.

Soluble Interfering Substance

The fixing process used in these studies extracts soaps and synthetic detergents from the bloodstain so that haemolysis due to these agents is not a problem. If oil is present the bloodstain should be washed in petroleum ether (b.p. 40°–60°C) before fixing. This solvent has no deleterious effect on ABO antigens and antibodies, and after several years of continuous use for grease extraction from bloodstains it is obvious that even prolonged extraction with petroleum ether has little or no effect on agglutinins. It should be emphasised that at the elevated temperatures used in the Absorption-Elution method, that the haemolytic power of any oil present in the system is increased. This can simply be observed by the use of Gorse (Ulex europaeus) extracts prepared from defatted and untreated seeds respectively. On causing these extracts to react with red cells at 50°C it is frequently noted that the untreated extract will haemolyse the red cells whereas the defatted extract will not.

When an undefatted Gorse extract is used as an 'antiserum' in the Absorption-Elution method it is often found that sufficient oil will survive the washing subsequently to be released on raising the temperature for elution. The indicator cells are thus haemolysed. A suitable preparation of Gorse extract is shown in Appendix D.

Insoluble Interfering Substance

Neither the fixing process nor extraction with Petroleum Ether will assist in the removal of water insoluble absorbing substances. These substances are of common occurrence in garments and often absorb anti A strongly but much less often anti B. Water soluble blood group substances such as occur in the saliva and semen of secretor individuals are extracted by the fixation process.

When the process of fixation by boiling was adopted we had hopes that most of the absorbing interfering substances which occur in clothing would prove to be water soluble. This, in our experience, has proved not to be the case and interfering absorption still occurs in some cases. However, it is uncommonly in a sufficient intensity to invalidate the results. We have carried out no experiments to determine the relative amount of insoluble and soluble absorbing substances but considering the common occurrence of soluble blood group substances in the body fluids of secretor type individuals then it is obvious that in at least some instances this method will reduce interfering absorption by extraction during the fixation process.

It is interesting to speculate on the nature of the insoluble absorbing substances commonly found in garments. It seems at least possible that some of this material will originate from exfoliated epithelial debris. The fact that we have on several occasions demonstrated blood group substances in finger nails by this method would tend to support this.

Assessment of Accuracy of the Absorption-Elution Method

The Absorption-Elution method has been used for some hundreds of tests in this laboratory and in several different variations two of which are described in my two notes (3, 4).

As an independent check on the method Dr. I. Dunsford of the Regional Blood Transfusion Laboratory, Sheffield, England, sent us 100 blood samples dried on filter paper without information as to their correct groups.

We grouped these stains in six batches of 15 stains and one batch of 10. Of these two batches of 15 were abandoned before completion (bloodstains no. 1–15 inclusive and 36–50 inclusive) owing to an influx of routine case work. Of the remaining 70 (35 x A_1, 16 x A_2, 2 x A_1B, 4 x A_2B, 7 x B, 6 x O) in 68 cases the correct agglutinogens were detected as confirmed by Dr. Dunsford whilst in the other 2 cases (both group A_2) no absorption was found. In both these cases however the blood samples were mainly serum and obviously originated from the supernatant of clotted specimens. It should be emphasised that in both these cases that failure to detect the A antigen would not have resulted in an error of reporting since as I have already emphasised we require proof of both agglutinogens and compatible agglutinins before we consider a bloodstain fully grouped in the ABO system. In both these cases our only positive finding was a β agglutinin and as such the bloodstain would have been reported merely as "A or O". Obviously to an experienced bloodstain grouper the detection of β but not α will immediately suggest group A and had there been sufficient cause we would doubtless have carried out further tests to detect A antigen.

The predominance of Group A in the tests reflects the fact that originally we had hoped to subgroup all these bloodstains in the A system but absorbing substances in the paper prevented this although it did not prevent main group determination.

Appendix A

The Detection of Agglutinins in Bloodstains

Each bloodstain to be grouped by the absorption method for agglutinogens should also be tested for agglutinins. A strip of bloodstained material about 1.0 cm. x 0.3 cm. is ideal for this purpose although with care tests can be made on smaller pieces.

Procedure. Cut the bloodstained strip in 3 squares each approx. 3 mm. x 3 mm. leaving the terminal 1 mm. strip for a precipitin test. Place each square at the bottom of an ignition tube (ca. 5 x 0.5 cm.) pressing with the tip of a Pasteur pipette until the fabric becomes slightly concave upwards. Next take 0.5% v/v cell suspensions of A_1, B and O cells and wet each piece of fabric with cell suspension using one piece each for A_1, B and O cells respectively. Place a piece of wet cotton wool adjacent to the tubes before covering with an inverted beaker.

The amount of cell suspension used must be the smallest possible commensurate with retrieving sufficient liquid for readings to be made. Leave for 3 hours then carefully pipette off the liquid, layer on a microscope slide and examine for agglutination.

It is permissible at this stage, if some evaporation has taken place, to add a small amount of saline to the tube, but this should not be a greater volume than the original volume of cell suspension.

Score intensity of agglutinations as in Dunsford and Bowley, *Techniques in Blood Grouping*, Oliver and Boyd, Edinburgh, 1954.

Interpretation of Results.

A_1	*B*	*O*	
+	+	−	Group O (neglecting atypical bloods such as A_4)
+	−	−	Anti A positive (i.e. Groups B or O)
−	+	−	Anti B positive (i.e. Groups A or O)
−	−	−	Nil Result.
+	+	+	Nil Result but seldom if ever encountered in blood stains
+	−	+	
−	+	+	
−	−	+	

Appendix B

Absorption-Inhibition Bloodstain Grouping in the ABO System

The following method of bloodstain grouping is adequate where the substratum of the stain is

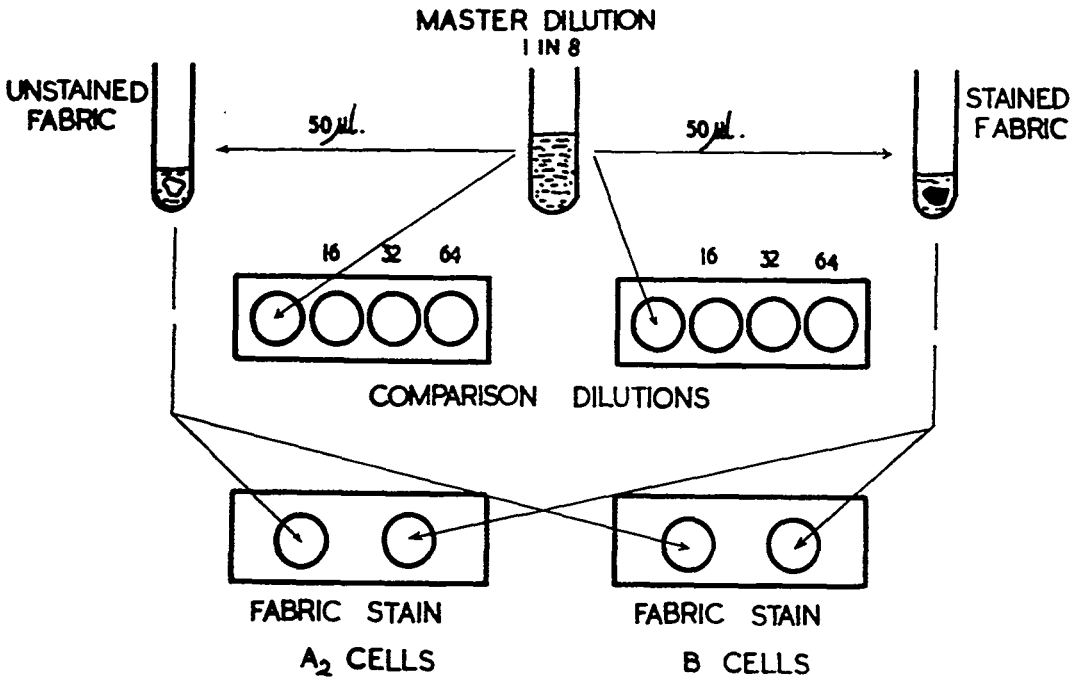

FIGURE 1

The above diagram is an example where the strength of the master dilution is 1/8. A preliminary titration should be made as follows to determine the correct strength of antiserum to use: Carry out two series of doubling dilutions of 1, 2, 4, 8, 16, 32, 64, 128, one for A_2 cells and the other for B. Choose the weakest reacting series, find the last place which gives good visual agglutinations and then use this as the master dilution. Cells should be used in 2% v/v concentration.

clean and nonabsorbing with respect to agglutinins. If the substratum is found to absorb then more complex techniques must be used. These will be described later.

Procedure. Cut out areas of 1 cm.^{2} of stained fabric and an immediately adjacent control of unstained fabric of the same size.

Cut the stained fabric as follows:

(a) A strip 0.3 mm. x 1.0 cm. for precipitin and agglutinin tests (See Appendix A).

(b) A strip 0.7 mm. x 1.0 cm. for absorption tests.

Cut the unstained fabric to the same area as the stained fabric. Take two round bottomed glass tubes of approximate size 1 cm. x 5 cm., scrupulously clean and with rubber (NOT CORK) bungs. Place the stained and unstained fabric separately in tubes and pipette 50 µl of suitably diluted Group O serum into each tube. Seal firmly and leave for a minimum of 3 hours at room temperature and then (preferably) complete the absorption by placing the tubes overnight in the refrigerator.

Next pipette off the absorbed antisera and dispense aliquots onto wax ringed slides. The exact value of an aliquot does not matter, but of course the chosen value must be absolutely constant. An average aliquot would be 15 µl which is sufficient for the necessary two of this size with an excess of 20 µl to allow for accidents and irrecoverable antisera. Add suitable cells, mix, and rotate gently in a moist chamber making a note of the order in which visible agglutination occurs. After 30 minutes note the final agglutination intensities.

When dispensing the absorbed antiserum onto slides a reference series of dilutions of the master dilution should also be made as shown in figure 1.

APPENDIX C

ABO BLOODSTAIN GROUPING WITH ABSORBING BACKGROUNDS

If, on attempting grouping of a blood (or semen or saliva) stain by the previous technique, it is found that the agglutinating power of an antiserum is wholly absorbed by both cloth and stain then the stain is most probably ungroupable. Some

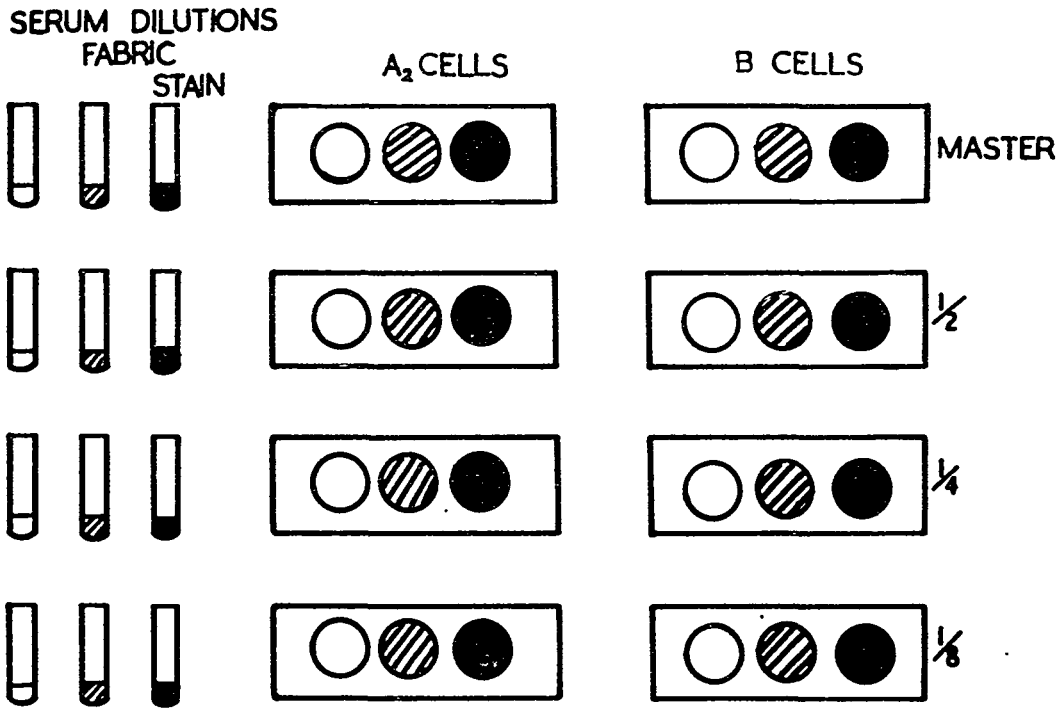

FIGURE 2

With fresh (say less than 3 days old) bloodstains and with saliva and seminal stains, extracts can be made and aliquots of extract titrated against the antiserum. Older bloodstains give poor results owing to the difficulty in obtaining a suitable extract which contains in solution and suspension the original blood group substances. Apart from the state of the bloodstain, however, (i.e. dry cloth or wet extract) the methods are the same. For a discussion on the advantages of carrying out absorption against aliquots of progressively weaker antiserum see Kind, *Vox Sanguinis* (1955).

information may be obtained however, by the agglutinin tests (Appendix A).

Should the circumstances warrant it, an attempt can be made to assess the relative amounts of absorbing substance in the stain and substratum. If it can be proved that the bloodstaining is consistently higher in say A substance than the adjacent cloth then the result is acceptable as proving that the original blood contained A substance.

In principle the test is precisely as in Appendix A the main differences being:

(a) Four dilutions of antiserum are used: the original master dilution and the three consecutive weaker dilutions.

(b) A piece of bloodstained fabric (and adjacent control) of the same size as the original test piece is cut into four equal pieces and four separate absorptions performed.

Figure 2 outlines these manipulations.

APPENDIX D

THE PREPARATION OF GORSE ANTI H LECTIN

Grind 2 g. dry Gorse (Ulex europaeus) seeds to a fine powder in a glass mortar. Add 25 ml. petroleum ether (B.P. 40°–60°) and regrind for 5 minutes. Allow to settle and discard the supernatant. Repeat with a further 25 ml. of petroleum ether. After all traces of solvent have volatilised add 20 ml. McIlvaine buffer, regrind and leave standing for 3 hours with occasional agitation. Spin and use the supernatant. The lectin can be conveniently stored as a slurry at 4°C in which case the supernatant is decanted with a Pasteur pipette as required.

For longer term storage it is best to allow the lectin to stand as a slurry in the refrigerator at 4°C for two to three days. Next spin and store the supernatant either frozen solid or after drying under reduced pressure over P_2O_5. (Reconstitute with distilled water.)

The period of storage at 4°C is to allow complete extraction and to allow for a precipitate which is thrown down from the supernatant especially in the first 24 hours.

McIlvaine buffer.

Citric Acid (monohydrate)	1.86	gm/litre
Na_2HPO_4 (anhydrous)	13.15	gm/litre
Thiomersalate	0.100	gm/litre

The titre of lectin against O cells when prepared by this method should be >7 (expressed as $\log_2$ dilution) for 15 minutes at 15°C on a glass plate against 2% cells.

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