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Glutaraldehyde as a Coupling Reagent in Passive Haemagglutination

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Summary. A sensitive passive haemagglutination technique is described depending on the attachment of proteins to erythrocytes with glutaraldehyde. Various factors that play a role in the coupling of bovine serum albumin to sheep erythrocytes are examined. A standard procedure was deduced by which other proteins were coupled and tested with their specific antisera. The observed titres are comparable to those obtained with the classical bis-diazotized benzidine method.

The technique is specific and practicable. It provides sensitized cells that remain stable for several weeks.

INTRODUCTION

The conjugation of proteins to erythrocytes has proved a valuable method for the detection of antigens and antibodies. In the different modifications of the haemagglutination technique, soluble antigens are linked to erythrocytes: (i) through adsorption as in Boyden's tanned cell technique (1951); or (ii) by stable covalences using bivalent reagents such as bis-diazotized benzidine (Stavitsky and Arquilla, 1958), 1,3-difluoro-4,6-dinitrobenzene (Ling, 1961a), tolylene-2,4-diisocyanate (Gyenes and Sehon, 1964) or 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (Johnson, Brenner and Hall, 1966). Erythrocytes treated with these reagents are frequently damaged to such an extent that haemolysis greatly interferes with subsequent titration. Therefore, erythrocytes are usually stabilized prior to sensitization by formaldehyde treatment (Ingraham, 1958). Ling (1961b) first mentioned glutaraldehyde as a stabilizing reagent. Moreover, he found glutaraldehyde-treated erythrocytes to be able to fix proteins from solution even without additional coupling reagent: this phenomenon was also observed by Bing, Weyand and Stavitsky (1967).

Our earlier observations on the polymerization of serum albumin and other proteins by glutaraldehyde (Joniau, Onkelinx and Lontie, 1968) led us to use this bifunctional reagent for linking proteins to erythrocytes.

In the present work the direct coupling of proteins to erythrocytes with glutaraldehyde was investigated. From the optimal conditions observed with the serum albumin system, a standard procedure was derived. The technique was further applied to other protein systems.

MATERIALS AND METHODS

Glutaraldehyde

Initial experiments were made with a suitably diluted, 25 per cent aqueous glutaraldehyde (GA)-solution (Fluka, Buchs, Switzerland). Afterwards, a 25 per cent GA-solution (Merck, Darmstadt, Germany) was purified by two charcoal treatments (20 g/100 ml).

The pure product obtained, showing a single absorbance maximum at 280 mμ (Fahimi and Drochmans, 1965), was titrated iodometrically according to Kolthoff and Belcher (1957). The final solution, diluted with water, was 0·25 M. Both preparations gave the same agglutination titre, but titrations with purified GA gave more distinct negative agglutination patterns.

Bis-diazotized benzidine

Bis-diazotized benzidine (BDB) was prepared according to Gordon, Rose and Sehon (1958). To a solution of 230 mg benzidine in 45 ml 0·2 N HCl were added 175 mg $NaNO_2$ dissolved in 5 ml distilled water. The reaction was allowed to proceed for 30 minutes at 0°, whereafter 1 ml fractions of the mixture were quickly frozen in a 2-propanol cooling bath at −35° and stored at −20°.

Prior to each experiment, the contents of a single vial were thawed and diluted fifteen-fold with 0·15 M phosphate buffer, pH 7·3. The final reagent contained 1·67 μmoles BDB/ml.

Sheep erythrocytes

Blood obtained in an abattoir was collected straight from the animal into an equal volume of Alsever's solution and stored for 1 or 2 weeks at 4°. However, most of the experiments were performed on a commercial preparation of washed, sterile cells (Nivelle, Wezembeek, Belgium). At their weekly arrival, they were centrifuged and resuspended in Alsever's solution (Campbell, Garvey, Cremer and Sussdorf, 1963). Before use the cells were washed three times with physiological saline (0·85 per cent NaCl, 0·008 per cent NaN_3).

Diluent

Pooled normal rabbit serum was decomplemented by heating for 30 minutes at 56° and diluted 100-fold with physiological saline.

Antisera

Rabbit antisera were prepared by weekly intramuscular injections of 10 mg antigen dissolved in 1 ml physiological saline and emulsified in Freund's complete adjuvant. After 6 weeks the rabbits were bled regularly at 2-week intervals. The injections were continued on the alternating weeks. The sera of consecutive bleedings were pooled for each rabbit. A goat antiserum against bovine serum albumin was obtained by a single bleeding after a series of intravenous injections.

The sera were heat-decomplemented, diluted with physiological saline and subsequently absorbed for 20 minutes with a volume of packed sheep cells equal to the undiluted antiserum.

Antigens

Bovine serum albumin (BSA) and transferrin (Tf) were prepared from oxalated plasma: after precipitation of the globulins with Na_2SO_4 (19 g/100 ml plasma) the supernatant was fractionated by gel filtration on Sephadex G-100 (Pharmacia, Uppsala, Sweden). The fraction containing BSA-monomers and some Tf was further purified by DEAE-cellulose chromatography. Tf was eluted in a first step using 0·02 M Tris–HCl buffer, 0·1 M NaCl at pH 8·0. BSA was then eluted by increasing the NaCl-concentration to 0·15 M. For the purpose of immunization, however, a commercial BSA-preparation was used (Armour, Chicago, U.S.A., lot No. P67908). β-Lactoglobulin A (βlg A) was prepared according to Aschaffenburg and Drewry (1957). Haemocyanin (Hmc) of *Helix*

pomatia was obtained by the method of Heirwegh, Borginon and Lontie (1961). Ovalbumin (OA) was prepared by the classical salt-fractionation method with $(NH_4)_2SO_4$ (Sørensen and Høyrup, 1915–17); the conalbumin (CA) was purified from the mother liquor of the OA-crystallization by repeated gel filtration on Sephadex G-100.

The purity of these different preparations was checked by agar-electrophoresis and agar-immunoelectrophoresis using the homologous antisera.

Sensitization with BDB

To 3 ml of protein solution (2·5 mg in 0·15 M phosphate buffer, pH 7·3) were added 0·1 ml of a 50 per cent suspension of erythrocytes in physiological saline and 0·5 ml BDB-solution (i.e. 8·3 μmoles). The mixture (total volume 3·6 ml) was left at room temperature for 15 minutes and centrifuged for 5 minutes. The cells were washed with 3·5 ml diluent and resuspended in 2·5 ml of the same (final concentration 2 per cent).

Sensitization with GA (standard procedure)

To 3·2 ml of protein solution (10 mg in 0·15 M phosphate buffer, pH 7·3) were added 0·1 ml of a 50 per cent cell suspension and 0·1 ml of 0·25 M GA-solution (i.e. 25 μmoles). After leaving the mixture (3·4 ml) for 1 hour at room temperature, the sensitized cells were centrifuged, washed with 3·5 ml diluent and finally resuspended in 2·5 ml to a final 2 per cent concentration.

Haemagglutination titration (standard procedure)

Two-fold dilutions of the antiserum were prepared with diluent and 0·5 ml aliquots transferred to a series of test tubes (11 × 75 mm). To each tube 0·05 ml of a 2 per cent suspension of sheep erythrocytes were added. The tubes were shaken vigorously and the titres read after settling for 3 hours. The titre was the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern. Slightly deviating titres were indicated with + or −. To each series of eight tubes constituting one titration was added a diluent-blank (0·5 ml diluent with 0·05 ml of sensitized cells) and an antiserum-blank (0·5 ml of the highest antiserum concentration with 0·05 ml of normal sheep erythrocytes).

Inhibition

To the series of increasing antiserum dilutions were added 0·05 ml inhibitor solution of constant concentration 30 minutes before mixing with the sensitized cells.

Calibration of antisera

The antibody content of antisera was estimated by quantitative precipitation using 10-μl aliquots. Nitrogen determinations on the precipitates were performed by the bromsulphalein precipitation technique of Glick, Good, Greenberg, Eddy and Day (1958). Calibration curves were linear up to 5 μg N.

RESULTS

INVESTIGATION OF THE COUPLING REACTION

In these experiments BSA has been coupled to sheep erythrocytes and tested with one individual rabbit antiserum (5b) for the sake of comparability. Optimal conditions have been selected in order to increase titres without losing specificity.

Effect of GA-concentration

Ten milligrams of BSA were coupled at constant reaction volume (3·4 ml) to 0·1 ml 50 per cent cell suspension using varying GA-quantities (Table 1). At high values (50–75 μmoles GA) non-specific agglutination frequently occurred which rendered end-point readings troublesome. Therefore 25 μmoles/3·4 ml coupling mixture were retained as the optimal value.

Table 1

Titres obtained for erythrocytes, sensitized with BSA by the standard GA-procedure: effect of the amount of GA on the coupling reaction

GA (μmoles)	Titre
2·5	50–100
7·5	400
12·5	400–800
25·0	800
50·0	1600+
75·0	1600+

Effect of BSA-concentration

The effect of BSA-concentration on the coupling reaction was measured at three different GA-levels. The changes occurring in the range from 2·5 to 40 mg BSA were minimal. Table 2 shows the best results for 25 μmoles of GA to range from 10 to 20 mg BSA.

Table 2

Titres obtained for erythrocytes, sensitized with BSA by the standard GA-procedure: effect of the amount of BSA on the coupling reaction

BSA (mg)	Titre at GA-level (μmoles)		
	7·5	25	75
1·0	<50	200	—
2·5	200–	400	12800–
5·0	200+	800+	6400+
10·0	200	800+	6400+
20·0	200–	1600	6400+
40·0	200–	800+	6400+
80·0	—	—	3200+

Effect of coupling time

Ten milligrams of BSA were coupled to erythrocytes by means of 25 μmoles GA, following the standard procedure. Samples of sensitized cells were taken at regular intervals. They were washed, suspended in diluent and used in haemagglutination titrations. As indicated by Table 3 the coupling reaction is virtually complete after 1 hour at room temperature.

TABLE 3

TITRES OBTAINED FOR ERYTHROCYTES, SENSITIZED WITH BSA BY THE STANDARD GA-PROCEDURE: EFFECT OF THE TIME ON THE COUPLING REACTION

Coupling time (minutes)	Titre
5	400
10	800
20	800–1600
30	800–1600
60	1600
120	1600–3200
240	1600–3200

Effect of pH

Ten milligrams of BSA were coupled to erythrocytes according to the standard GA-procedure in 0·15 M phosphate buffers at different pH and also in physiological saline (Table 4). The best titres were obtained in the pH-range from 7 to 8. A pH-value of 7·3 or 7·4 was chosen as optimal.

TABLE 4

TITRES OBTAINED FOR ERYTHROCYTES, SENSITIZED WITH BSA BY THE STANDARD GA-PROCEDURE: EFFECT OF pH ON THE COUPLING REACTION

pH	Titre
5·2	400
6·1	800
6·7	800–1600
7·4	1600
8·1	1600+
Physiological saline (pH 6·0)	800

SELECTIVITY OF THE GA-METHOD

The selectivity of haemagglutination titrations carried out with GA-sensitized cells was checked by inhibition with the corresponding antigen (BSA). Several titrations were performed each with a different inhibitor concentration. Table 5 shows the titre to decrease with the amount of BSA added initially. The 'inhibition' by ovalbumin served as a blank: this caused no change in titre.

TABLE 5

PASSIVE HAEMAGGLUTINATION TITRATION OF RABBIT ANTI-BSA ANTISERUM WITH BSA-COATED CELLS SENSITIZED BY THE STANDARD GA-PROCEDURE: INHIBITION WITH VARYING AMOUNTS OF BSA AND OA

Inhibitor (μg/tube)		Titre
BSA	0·0	3200
	0·1	800
	1·0	50–100
	10·0	<25
OA	10·0	3200

SENSITIVITY OF THE GA-METHOD

In order to show a relationship between the observed titre and the antibody content of antisera, three different rabbit antisera against BSA were compared by quantitative precipitation analysis and by haemagglutination titration following the standard GA-procedure (Table 6).

TABLE 6
ANTIBODY CONTENT OF DIFFERENT RABBIT ANTI-BSA ANTISERA COMPARED WITH HAEMAGGLUTINATION TITRES FOLLOWING THE STANDARD GA-METHOD

Antiserum	Precipitin content (mg/ml)	Titre
5a	0·56	1600+
57	1·34	12800+
50	3·76	51200

STABILITY OF GA-SENSITIZED CELLS

Treatment of the erythrocytes with GA in the sensitizing step resulted, in addition to antigen coupling, in a fixation of the cell membranes to such an extent that subsequent haemolysis was avoided for a period of several weeks when the sensitized cells were stored at 4° in diluent. Haemagglutination titres, determined after a month's storage of the sensitized cells, paralleled initial values (Table 7).

TABLE 7
STABILITY OF GA-SENSITIZED CELLS AFTER STORAGE AT 4° IN DILUENT

Antigen	Titre	
	Initial	After 1 month
BSA	51200	12800–25600
βlg A	6400+	1600–3200

APPLICATION TO DIFFERENT ANTIGEN–ANTIBODY SYSTEMS

The standard GA-method of sensitizing sheep erythrocytes was applied to other antigen–antibody systems and each of them compared with the standard BDB-method (Table 8). By inhibition with homologous antigen the selectivity of several systems was checked.

DISCUSSION

Of the various factors that play a role in the coupling reaction with glutaraldehyde, by far the most important are GA-concentration, reaction volume, reaction time and pH. Changes in the total amount of BSA (from 2·5 to 40 mg/3·4 ml) and of temperature (4° or 20°) have little effect on the final readings. The titre increases with the amount of GA used, but at higher values non-specific agglutination patterns appear. A reaction time of 1 hour is found sufficient for optimal coupling to occur.

TABLE 8
TITRES OBTAINED FOR ERYTHROCYTES, SENSITIZED WITH DIFFERENT ANTIGENS BY MEANS OF THE STANDARD BDB- AND GA-METHODS

Antigen	Antiserum	Inhibitor	Titre	
			BDB	GA
BSA	Anti-BSA (5a)	—	3200 –	3200 +
	Anti-BSA (5b)	—	1600	3200
	Anti-BSA (5b)	10 μg BSA	—	<25
	Anti-BSA (5b)	10 μg OA	—	3200
	Anti-BSA (50)	—	25600–51200	51200
	Anti-BSA (goat)	—	800 +	1600–3200
Tf	Anti-BSA (goat)	—	100	200
βlg A	Anti-βlg A	—	3200 –	12800
	Anti-βlg A	10 μg βlg A	—	100
OA	Anti-OA (6N)	—	320000 +	12800–25600
	Anti-OA (22N)	—	320000	12800 +
	Anti-OA (22N)	10 μg OA	—	200
	Anti-OA (22N)	10 μg CA	—	12800
CA	Anti-OA (22N)	—	40000 +	40000 +
Hmc	Anti-Hmc_{tot}	—	160000 +	160000
	Anti-Hmc_{α}	—	80000 +	80000–160000

The influence of the total reaction volume is as expected: the more dilute the coupling medium, the lower are the titres. The pH-effect on the other hand is at variance with our previous observations on the polymerization of BSA. This proceeds optimally in the lower pH-range (4–5), in agreement with the known acid catalysis of the reaction between amino-groups and aldehydes. In haemagglutination, however, results were optimal at pH 7–8.

An insight was gained in the stability of the GA–protein bonds by referring to our previous experience with BSA-polymers obtained with the same coupling agent. A pure preparation of dimers, isolated by gel-filtration and subjected to the action of different media, was checked for redissociation by Sephadex chromatography; neither slightly basic, nor moderate acid conditions (0·1 M HCl) had any pronounced effect.

The sensitivity of the agglutination method described was evident on comparison of the titres observed under standard conditions with the actual precipitin content: a correlation was observed.

In addition to BSA, the standard GA-procedure was applied to other antigen systems and at the same time compared with the classical BDB-method (Stavitsky and Arquilla, 1958). GA-sensitization resulted in at least as high a titre for bovine serum albumin, transferrin, conalbumin and haemocyanin. For ovalbumin the method was less sensitive, whereas for β-lactoglobulin A it proved to give very high titres in contrast with the findings of Bing *et al.* (1967) who were unable to detect any βlg A adsorption onto GA-coated cells.

The specificity of the method was demonstrated by inhibition tests in the case of serum albumin, β-lactoglobulin and ovalbumin. When investigating new antigens, the optimal conditions originally deduced for BSA were applied. It might be possible to obtain higher titres by redetermining the optimal conditions for each individual protein.

The fact that anti-BSA antisera consistently gave a Tf-precipitin line on immunoelectrophoresis when tested with whole serum, indicated that the commercial preparation used for injection might still contain traces of transferrin. Moderate titres for anti-transferrin

were found indeed when anti-BSA antisera were titrated with transferrin-coated cells. On the other hand, high titres for anti-conalbumin were recorded with anti-OA antisera. The haemagglutination reaction of OA-sensitized cells and anti-OA antisera however proved to be entirely specific, as no inhibition occurred even at high levels of CA. This is another disproof of the older claims, based on tannic acid sensitization, that CA might obscure OA titration (Borduàs and Grabar, 1953).

When comparing the method described with the classical BDB-procedure, some points of interest have to be stressed. GA-coupling leaves very stable sensitized cells, not subject to subsequent haemolysis. These cell preparations could be used for several weeks without a gross change in titre.

A real advantage is the fact that GA reacts nearly exclusively with amino-groups. This allows for proteins or peptides with no or blocked tyrosine and histidine-groups to be coupled. Thus, in preliminary experiments the GA-method was used for titrating anti-hapten antisera. Sulphanilated BSA was coupled to sheep erythrocytes and the sensitized cells were used to titrate sheep antisera against sulphanilated haemocyanin. BDB-coupling led to negative results as was expected, whereas with GA-sensitized cells we succeeded in quantitating our anti-hapten antisera.

The GA-method readily produces sensitized cells that give specific and reproducible haemagglutination titrations, and that remain stable for long periods. Furthermore it allows the coupling of protein material lacking tyrosine.

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