Chapter 6 Effect of blocker

6.1 Introduction

Solid phase immunoassays are sensitive and reliable (Tijssen,1985: Aldao and Vides,1984). Such properties are strongly dependent upon the concentration of the ligand adsorbed on to the solid phase immobilised with binder protein (Pesce et al., 1981; Makela & Peterfy, 1983) and the non-specific binding of other proteins components in the sample (Bjercke et al.,1986; Sarma et al., 1986). Solid phase immunoassays often involve proteins (antibodies / antigen) immobilised to a polymer surface by non-specific binding (NSB). However, non-specific binding of other protein components during the subsequent steps of such assays is detrimental to their sensitivity and specificity. Engvall and Perlmann (1972) found the non-ionic detergent Tween 20 to be an effective blocking agent when added to dilution and washing buffers. With the widespread application of solid phase immunoassay for antigen and antibody determination a number of modifications have been adopted to obtain a satisfactory reduction in background readings. These include addition of proteins to dilution and washing buffers in combination with Tween 20 alone (Ruitenberg et al., 1974, 1976) as well as an extra protein coating aimed at saturating the polymer support not occupied by antigen prior to probing it with antibodies (Mathiesen et al., 1978; Naot et al., 1981). The undesirable non-specific binding may be minimised by saturating the substrate 's remaining adsorption

surface with 'blocking proteins', a collective term used for various protein additives that have no active part in the immunochemical reaction of the assays. Blocking with tris(hydroxymethyl) aminomethane, ethanolamine, and gelatin have proved to be effective in reducing or eliminating non-specific binding reactions (Renart et al., 1979). Others blocking agents used include BSA (Towbin et al., 1979), haemoglobin (Gershoni and Palade, 1982), gelatin (Lim and Kasamatsu, 1983), and milk (Johnson et al., 194). However, the choice of blocking agents to inhibit NSB is critical to the sensitivity and specificity of an immunoassay system (Spinola and Cannon, 1985; Bjercke et al., 1986; Sarma et al., 1986). The blocking agents and conditions used will depend on the type of solid phase and sensitivity of the system needed. The aim of this experiment is to seek suitable blockers that can reduce non-specific binding of the WNR surface effectively.

6.2 Materials and experimental methods

6.2.1 Materials - Please refer to Section 2.2 and 5.2

6.2.2 Experimental methods

NR coated tube was pre washed five times with 1 ml of 0.1 M HCl followed by five washes with 1 ml of distilled water, then coated with anti-HBs or HBsAg as described in Section 2.2. 200 µl of blocker were added into each tube, and left to equilibrate overnight at 4°C. The solution was decanted and the tubes were washed

four times with 1 ml of PBS solution. To reblock, the above process was repeated according to the number of reblockings required. The tubes are now ready for assay as described in Section 2.2.

6.2.3 Determination of precoating concentration

Total protein concentration of 50% NBCS and NEO were determined using the DuPont Dimension system. The total protein concentration of 50% NBCS and NEO were 19.6 g dm^{-3} and 52.8 g dm^{-3} respectively.

6.3 Results

Most of the blockers tested were able to reduce non-specific binding to different extents. On WNR surface immobilised with anti-HBs, increasing concentration of gelatin (used for single blocking) reduced non-specific binding (without serum incubation) (Table 6.1). However changes in BSA concentration (used for single blocking) did not show significant changes in non-specific binding (without serum). Increasing the number of blocking steps to three for all blockers (Fig. 6.1a) reduced the non-specific binding (with or without serum) of ^{125}I anti-HBs on anti-HBs immobilised tube. However for gelatin and NEO (without serum), optimum condition was achieved after reblocking for three times, whereas NBCS & BSA further reduced non-specific binding up to five reblockings. By repeating the blocking process, the results clearly show that NBCS and NEO (without-serum Fig

6.1a) are more effective blockers in reducing non-specific binding compared to gelatin and BSA. In the presence of negative control serum, non-specific binding was further reduced after five reblockings except in the case of 0.5% BSA & NEO. The efficiency of the blockers is in the order NBCS=NEO>BSA=gelatin (Fig 6.1a).

The specific binding of ^{125}I anti-HBs for NBCS, NEO and BSA was lower compared to gelatin when they were used as blockers (Fig. 6.1b). Gelatin seemed to give the highest specific binding. No specific trend on changes of the specific binding with the number of reblockings was observed. In general anti-HBs immobilised tube with lower non-specific binding (in the presence of HBsAg negative serum), would give higher specific binding irrespective of the number of reblockings. Generally reblockings reduced specific binding except NBCS, which showed an increase. Specific binding was reduced to the greatest extent by blocking with 0.5% gelatin (Fig. 6.1c)

For WNR tube immobilised with HBsAg, increasing the concentration of gelatin and BSA (used for single blocking), caused a decrease in non-specific binding (with or without serum) (Table 6.2, Fig. 6.2a). Reblocking also showed a general decreasing trend of non-specific binding and the results also indicated that NEO and NBCS were more effective blockers than gelatin and BSA (Table 6.2, Fig. 6.2a). 1.5% of gelatin gave the highest specific binding of ^{125}I HBsAg, it was then followed by NEO. NBCS give lowest specific binding of ^{125}I HBsAg (Fig. 6.2b). The

specific binding of ^{125}I HBsAg decreased with the number of re-blockings except for 0.5% BSA (Fig. 6.2c).

For PP tube immobilised with anti-HBs and in the absence of negative control serum, except NBCS & BSA, non-specific binding was reduced as the number of re-blockings increased (Table 6.3). Similarly, specific binding of ^{125}I anti-HBs was reduced concurrently for all blockers as the number of re-blockings increased (Table 6.3).

As for PP tube immobilised with HBsAg, reblocking did not show significant changes in non-specific binding but specific binding was reduced when BSA & NEO were used as blockers after reblockings. However specific binding was enhanced slightly after five blockings with gelatin and NBCS (Table 6.4).

Table 6.1 Effect of blocker on ^{125}I anti-HBs binding by WNR surface (in HBsAg assay)
WNR coated tube immobilised with anti-HBs, blocked with different blockers and incubated in positive HBsAg serum or negative HBsAg serum or without serum incubation. ^{125}I anti-HBs was then added and incubated again. The tube was washed and the bound radioactivities were counted. WNR tube = NR coated tube pre washed five times with 1 ml of 0.1 M HCl and followed by five washes with 1 ml of distilled water.
*The numbers indicated in all the tables are the mean of three determinations together with their standard errors. This applies to all the Tables in the Chapter.

	* Percent binding				
Blocker, no of blocking	With HBsAg (Positive serum)(*a)	With HBsAg (Negative serum) ('b)	'Without serum	Specific binding % (a-b)	a/b
1.5% gelatin (1x)	4.27±0.22	1.83±0.18	2.93±0.01	2.44	2.33
1.0% gelatin (1x)	4.83±0.08	1.82±0.17	3.12±0.08	3.01	2.65
0.5% gelatin (1x)	4.95±0.43	2.15±0.04	3.19±0.10	2.80	2.30
0.5% gelatin (3x)	3.70±0.30	2.03±0.10	2.94±0.01	1.67	1.82
0.5% gelatin (5x)	3.87±0.34	1.90±0.05	2.94±0.09	1.97	2.04
1.5% BSA(1x)	3.01±0.38	2.03±0.08	2.84±0.06	0.98	1.48
1.0% BSA (1x)	3.00±0.08	2.01±0.10	2.87±0.08	0.99	1.49
0.5% BSA (1x)	3.17±0.28	1.92±0.16	2.88±0.11	1.25	1.65
0.5% BSA (3x)	3.11±0.25	1.91±0.19	2.62±0.08	1.20	1.63
0.5% BSA (5x)	3.18±0.28	1.96±0.08	2.00±0.34	1.22	1.62
NEO (1x)	3.12±0.11	2.02±0.16	2.46±0.07	1.10	1.54
NEO (3x)	3.18±0.08	1.69±0.23	1.79±0.16	1.49	1.88
NEO (5x)	2.87±0.13	1.79±0.29	1.93±0.03	1.08	1.60
NBCS (1x)	2.97±0.21	2.10±0.09	2.21±0.08	0.87	1.41
NBCS (3x)	2.83±0.13	1.83±0.20	1.91±0.09	1.00	1.54
NBCS (5x)	2.78±0.32	1.73±0.00	1.83±0.08	1.05	1.61

Table 6.2 Effect of blocker on ^{125}I HBsAg binding by WNR surface (in anti-HBs assay)
WNR coated tube immobilised with HBsAg and blocked with different blockers and incubated in positive anti-HBs serum or negative anti-HBs serum or without serum incubation. ^{125}I HBsAg was then added and incubated again. The tube was washed and the bound radioactivities were counted. WNR tube = NR coated tube pre washed five times with 1 ml of 0.1 M HCl and followed by five washes with 1 ml of distilled water.

	Percent binding				
Blocker, no of blocking	With anti-HBs (Positive Serum)(a)	With anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
1.5% gelatin (1x)	2.51±0.28	0.35±0.01	0.35±0.02	2.16	7.17
1.0% gelatin (1x)	1.87±0.27	0.37±0.03	0.42±0.03	1.50	5.05
0.5% gelatin (1x)	1.85±0.07	0.38±0.01	0.42±0.02	1.47	4.87
0.5% gelatin (3x)	1.54±0.31	0.35±0.03	0.42±0.03	1.19	4.40
0.5% gelatin (5x)	1.47±0.18	0.39±0.01	0.30±0.03	1.08	3.77
1.5% BSA(1x)	1.92±0.38	0.32±0.01	0.37±0.00	1.60	6.00
1.0% BSA (1x)	1.92±0.14	0.35±0.01	0.40±0.01	1.57	5.49
0.5% BSA (1x)	1.94±0.26	0.38±0.01	0.37±0.10	1.56	5.11
0.5% BSA (3x)	1.62±0.42	0.34±0.02	0.38±0.06	1.28	4.76
0.5% BSA (5x)	1.69±0.19	0.35±0.04	0.36±0.01	1.34	4.83
NEO (1x)	2.14±0.39	0.36±0.03	0.39±0.02	1.78	5.94
NEO (3x)	1.67±0.32	0.34±0.01	0.32±0.03	1.33	4.91
NEO (5x)	1.46±0.42	0.27±0.03	0.31±0.04	1.19	5.41
NBCS (1x)	1.77±0.05	0.34±0.01	0.38±0.01	1.43	5.20
NBCS (3x)	0.88±0.06	0.18±0.02	0.28±0.03	0.70	4.89
NBCS (5x)	0.82±0.03	0.16±0.02	0.26±0.03	0.66	5.12

Table 6.3 Effect of blocker on ^{125}I anti-HBs binding by PP surface (in HBsAg assay)
PP tube immobilised with anti-HBs and blocked with different blockers and incubated in positive HBsAg serum or negative HBsAg serum or without serum incubation. ^{125}I anti-HBs was then added and incubated again. The tube was washed and the bound radio activities were counted..

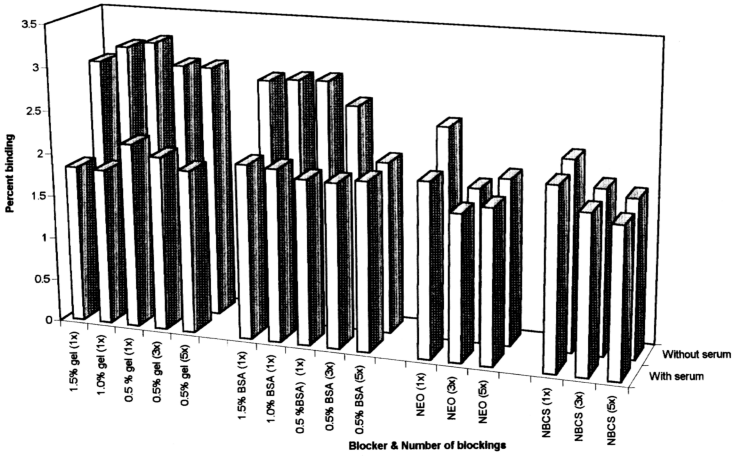
	Percent binding				
Blocker, no of blocking	With HBsAg (Positive serum)(a)	With HBsAg (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
1.5% gelatin (1x)	2.11±0.17	0.14±0.01	0.27±0.01	1.97	15.07
1.0% gelatin (1x)	1.91±0.16	0.17±0.02	0.20±0.03	1.74	11.23
0.5% gelatin (1x)	2.22±0.18	0.15±0.03	0.19±0.02	2.07	14.80
0.5% gelatin (5x)	1.96±0.08	0.17±0.01	0.12±0.01	1.79	11.53
1.5% BSA(1x)	2.19±0.22	0.16±0.02	0.13±0.02	2.03	13.69
1.0% BSA (1x)	1.70±0.06	0.14±0.01	0.13±0.01	1.56	12.14
0.5% BSA (1x)	1.45±0.21	0.13±0.01	0.13±0.01	1.32	11.15
0.5% BSA (5x)	1.43±0.40	0.22±0.11	0.15±0.11	1.21	6.50
NEO (1x)	1.12±0.24	0.14±0.02	0.15±0.02	0.98	8.00
NEO (5x)	0.86±0.06	0.16±0.02	0.10±0.02	0.70	5.37
NBCS (1x)	1.88±0.06	0.20±0.01	0.14±0.01	1.68	9.40
NBCS (5x)	1.83±0.21	0.20±0.01	0.18±0.01	1.63	9.15

Table 6.4 Effect of blocker on ^{125}I HBsAg binding by PP surface (in anti-HBs assay)
PP tube immobilised with HBsAg and blocked with different blocker and incubated in positive anti-HBs serum or negative anti-HBs serum or without serum incubation. ^{125}I HBsAg was then added and incubated again. The tube was washed and the bound radioactivities were counted.

	Percent binding				
Blocker, no of blocking	With anti-HBs (Positive serum)(a)	With anti-HBs (Negative serum) (b)	Without serum	Specific binding % (a-b)	a/b
1.5% gelatin (1x)	12.18±1.68	0.17±0.02	0.12±0.02	12.01	71.65
1.0% gelatin (1x)	12.31±0.29	0.18±0.01	0.13±0.01	12.13	68.33
0.5% gelatin (1x)	13.30±0.92	0.17±0.04	0.13±0.07	13.13	78.23
0.5% gelatin (5x)	13.78±1.34	0.15±0.03	0.10±0.02	13.63	91.87
1.5% BSA(1x)	13.61±0.89	0.15±0.15	0.15±0.01	13.46	90.73
1.0% BSA (1x)	12.44±0.51	0.16±0.16	0.16±0.01	12.28	77.75
0.5% BSA (1x)	12.58±1.05	0.16±0.01	0.20±0.01	12.42	78.62
0.5% BSA (5x)	11.90±1.29	0.22±0.05	0.23±0.03	11.68	54.09
NEO (1x)	12.05±0.31	0.27±0.01	0.26±0.05	11.78	44.63
NEO (5x)	11.23±1.46	0.53±0.08	0.46±0.12	10.70	21.19
NBCS (1x)	12.76±1.48	0.22±0.01	0.18±0.06	12.54	58.00
NBCS (5x)	13.22±0.39	0.20±0.03	0.16±0.02	13.02	66.10

Fig.6.1a Effect of blocker on non- specific binding of ^{125}I anti-HBs by WNR tube (in HBsAg assay)

WNR coated tube immobilised with anti-HBs , blocked with different blocker and preincubated with HBsAg negative control serum or without serum incubation before ^{125}I anti-HBs was added. The tube was washed and the bound radioactivities were counted. WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water.



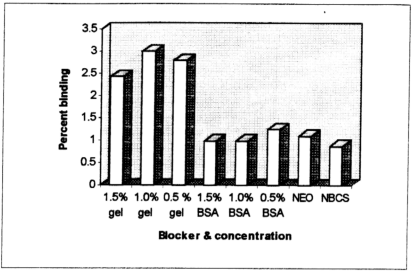


Fig. 6.1b Effect of blocker on specific binding of ^{125}I anti-HBs by WNR tube (in HBsAg assay)
WNR coated tube immobilised with anti-HBs , blocked with different blockers and preincubated with HBsAg positive control serum or HBsAg negative control serum before ^{125}I anti-HBs was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in positive control serum - percent binding in negative control serum . WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water. gel = gelatin

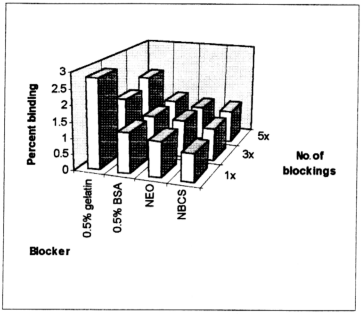
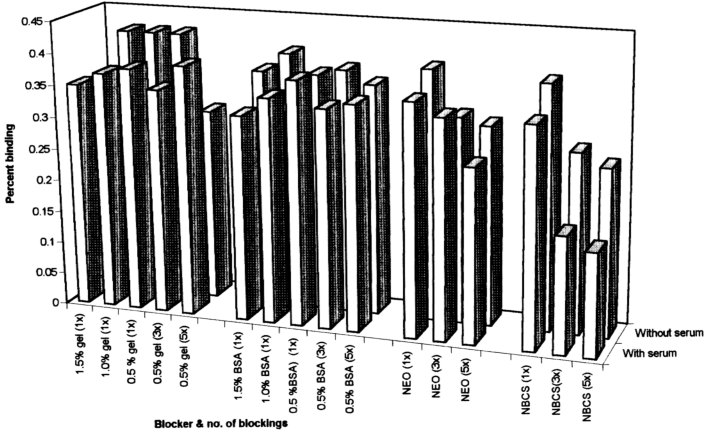


Fig. 6.1c Effect of blocker on specific binding of ^{125}I anti-HBs specific binding by WNR coated tube (in HBsAg assay)

WNR coated tube immobilised with anti-HBs , blocked with different blocker to the numbers of times required and preincubated with HBsAg positive control serum or HBsAg negative control serum before ^{125}I anti-HBs was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in positive control serum - percent binding in negative control serum. WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water.

Fig.6.2a Effect of blocker on non- specific binding of ^{125}I HBsAg by WNR tube (anti-HBs assay) WNR coated tube immobilised with HBsAg , blocked with different blocker and preincubated with anti-HBs negative control serum or without serum incubation before ^{125}I HBsAg was added. The tube was washed and the bound radioactivities were counted. WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water. NSB (serum) - non specific binding with negative control serum; NSB (without serum) - non specific binding without serum preincubation.



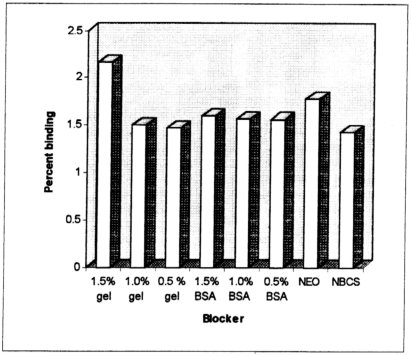


Fig. 6.2b Effect of blocker on specific binding of ^{125}I HBsAg by WNR tube (in anti-HBs assay)
WNR coated tube immobilised with HBsAg, blocked with different blockers and preincubated with anti-HBs positive control serum or anti-HBs negative control serum before ^{125}I HBsAg was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in positive control serum - percent binding in negative control serum . WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water. Gel = gelatin

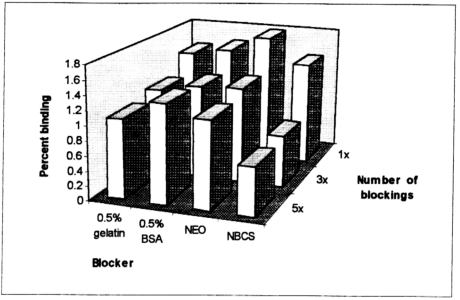


Fig. 6.2c Effect of blocker on specific binding of ^{125}I HBsAg by WNR coated tube (in anti-HBs assay)

WNR coated tube immobilised with HBsAg. blocked with different blockers to the number of times required and pre incubated with anti-HBs positive control serum or anti-HBs negative control serum or before ^{125}I HBs was added. The tube was washed and the bound radioactivities were counted. Specific binding = Percent binding in positive control serum - percent binding in negative control serum . WNR tube = NR coated tube pre washed five times with 1 ml of 0.1M HCl and followed by five washes with 1 ml of distilled water.

6.4 Discussion

Vogt et al (1987) showed that instantized dry milk, casein, gelatin from porcine and fish skin, skin albumin and several other proteins were able to block non-specific binding (NSB) of a peroxidase-conjugated immunoglobulin to polystyrene (PS) microtiter plate wells. Each blocking protein was tested across a million-fold concentration range, both in simultaneous incubation with the peroxidase conjugate and as a pretreatment agent where excess protein was washed away before incubation with conjugate. It was found that the concentration of each protein solution required for maximum inhibition was above 0.1% w/v. Therefore gelatin and BSA concentrations used for this work at 0.5% w/v were well above this level. To study the efficacy of blocking agents in preventing non-specific binding, a simple system was used to measure binding of labelled protein on to anti-HBs and HBsAg immobilised tubes which have been saturated by different blockers. Its inhibition by the different blocking solutions is shown in Table 6.1 - 6.4.

Vogt et al (1987) suggested two mechanisms of reduction of non-specific binding of polymer surface by blockers. Some protein (such as casein) blocked non-specifically through protein-polymer interactions, while others (such as porcine skin gelatin) blocked primarily through protein-protein interactions. Protein-polymer interaction depends on the proportion of soluble protein that binds to the polymer surface. One of the examples tested was casein which maintained a consistent 92-94% inhibition through NSB when solution containing as little as 400 ng was used to pretreat polystyrene microtitre plate. (Pretreatment procedure - PS plates were

first incubated with dilution of various blocking proteins for 1 hour at 37 °C. After washing, the wells were incubated for one hour with goat anti-mouse peroxidase conjugate, and the PS bound peroxidase activity was then determined). This gave the range of minimal saturation by protein on polymer surface during incubation. (The amount of protein, even if entirely bound to the polymer, was just in the range of minimal saturation for the polymersurface exposed during incubation. The rapid loss of blocking activity was seen when less than 400 ng was used, and this was commensurate with protein levels falling below the theoretical saturation range.) From the results obtained in this study, it was concluded that NBCS and NEO were the most effective blockers for WNR surface. One of the reasons NBCS and NEO sera have high blocking effect was the presence of proteins with different sizes. As revealed by scanning electron micrographs and AFM pictures (Fig. 2.24 - 2.27), NBCS almost covered up the unoccupied immobilised substrate surface. Adsorption of NEO on WNR (Fig. 5.11 a & b) surface shows feature similar to that of NBCS (Fig. 2.19), 0.5% of gelatin and BSA (Fig. 5.7 & 5.9 a & b) adsorbed on the surface as cluster and did not cover the WNR surface evenly. After anti-HBs or HBsAg immobilisation followed by blocking with BSA or gelatin, considerable fraction of the substrate remained . Thus these were not able to cover the substrate surface as effective as NEO and NBCS and hence the reduction of non-specific binding was also less effective. The protein concentration of NBCS, NEO, 0.5% BSA and 0.5% gelatin used were 19.6 g dm^{-3}, 52.8 g dm^{-3}, 5 g dm^{-3} and 5 g dm^{-3} respectively. It was found that blocking was dependent on the protein

concentration and the ability of the blocker to form evenly spread layer which was observed when NEO & NBCS were used as blockers. The latter will prevent non-specific adsorption of labelled anti-HBs or HBsAg onto WNR solid phase.

However NBCS and NEO could result in the desorption of bound anti-HBs or HBsAg to a higher degree than other blockers such as gelatin and BSA (Table 7.2). Anti-HBs and HBsAg adsorbed on to the surfaces may be desorbed when a second adsorption step utilising different protein was carried out. According to Vroman & Adams (1985) , α-globulins were more effective in displacing proteins from silica surface than albumins. It was also found that the initially immobilised IgG could be displaced by proteins in the solutions, and that the displacement was a statistical process which depends strongly on the degree and mode of structural changes occurred on adsorption. Displacement of the immobilised IgG by proteins in solutions was dependent on both the proteins used and the surface to which IgG was adsorbed (Bale et al, 1989). The general rule is that the higher the initial IgG concentration, the easier the adsorbed IgG could be displaced progressively. These results suggest that the degree of crowding of adsorbed IgG at the surface influences the structural arrangement occurring as assessed by the ease of displacement.Similarly, NEO and NBCS at higher concentrations should be able to displace the immobilised proteins more effectively. As a result, the a/b ratio was comparatively low (Table 6.2).

For both WNR and PP surfaces in general, reblocking reduced non-specific binding as shown by Table 6.1 -6.4. NR surface immobilised with anti-HBs or

HBsAg and blocked with NBCS appeared to be fully covered by the NBCS protein molecules (Fig. 2.24 & 2.25). As discussed in Chapter 3, exchange of molecules could have occurred here. NBCS molecules loosely bound over a certain area could be displaced by labelled molecules from the solution. Reblocked molecules were adsorbed more strongly on to the substrate surface, as their arrangements were more compact and were not easily desorbed from the surface . On WNR surface immobilised with HBsAg, optimal specific binding was achieved after three blockings in the presence of serum (except for gelatin). In anti-HBs coated tube, non-specific binding (in the presence or absence of negative serum) was further reduced by five blockings (except 0.5% BSA and NEO) in the presence and absence of negative serum. The smaller size ^{125}I anti-HBs seem to be able to penetrate though the immobilised proteins to the substrate below easily and non-specific binding could be reduced by increasing the number of blockings to five times. Even though reblocking reduced non-specific binding, for each blocking step , the blockers would displace some immobilised protein. Thus anti-HBs or HBsAg proteins immobilised on the surface would be reduced and thus decreasing the sensitivity of the assay.

In protein-protein interaction, the blocker in solution interacts with the adsorbed protein molecules on the polymer surface to provide an effective barrier against non-specific binding. This type of blocker serves as a prototype for low avidity, poor coverage blocking protein. Such agents would be expected to function poorly in the pretreatment protocol (See pg. 271) since the weak protein-protein interactions

could not prevent disruption of the multi-layered molecular barrier when the solid phase was washed. For example, hydrolysed gelatin (Hygel) (Kato et al., 1980), the least effective blocking agent tested, could not reduce NSB by more than 50% in the pretreatment protocol, even at concentration that would ensure complete saturation of the polymer surface. In contrast, Hygel was able to reduce NSB by almost 90% in the simultaneous incubation protocol (Simultaneous incubation protocol - PS plates were incubated for 1 hour with a solution containing both goat anti-peroxidase conjugate and the blocking solution at 37°C, PS-bound peroxidase activity was then determined). This observation suggests that excess Hygel in solution interacted with the protein molecules adsorbed onto the polymer surface to provide more effective barrier against NSB. BSA was found to do well in pretreatment protocol where the coated solid phase is saturated with blocking protein before it was used to carry out the assay (Vogt et al.,1987). Using BSA as a blocker for WNR tube immobilised with anti-HBs or HBsAg, the non-specific binding (in the absence of negative control serum) did not change significantly with increasing concentration of BSA concentration. However, with an increase in BSA concentration in the presence of serum, NSB were reduced significantly for ^{125}I HBsAg binding. This behaviour was not observed when gelatin was used as a blocker which gave a decreasing trend of non-specific binding (in the presence or absence of negative control serum) with increasing concentration of gelatin, of both ^{125}I anti-HBs & ^{125}I HBsAg. However with tube incubated with serum, reblocking with 0.5% BSA did not significantly reduce the non-specific binding of ^{125}I anti-

HBs but non-specific binding of ^{125}I HBsAg was decreased. BSA was expected to interact with protein in the serum, forming complex which was able to further reduce non-specific binding more effectively.

Increase in gelatin concentration reduced the non specific binding on both anti-HBs and HBsAg immobilised WNR surface (with or without serum). But this behaviour was not observed in BSA. Vogt et al (1987) also found that BSA retained maximal blocking activity between concentration of 0.31 - 2.50 % w/v on polystyrene microtitire plate. Inhibition effect decreased gradually when the concentration decreased from 0.31% (w/v) to a minimum value of $0.16x10^{-3}$ % w/v. It is possible that 0.5% of BSA was within the optimal concentration range for reducing the non specific binding in the present system. Gelatin, a heterogeneous mixture of water-soluble protein with high molecular weight was the least effective blocking agent. It is not found in nature but derived from collagen by hydrolytic action of boiling skin, tendons, ligaments and bones. Vogt et al (1987) also indicated that Porcine skin gelatin showed maximum inhibition at concentration between 0.62-2.5% w/v. At concentration lower these 0.62 % w/v the inhibition effect was reduced. Our results which show that 0.5% gelatin w/v gave the lowest inhibition of non-specific binding in the absence of negative control serum were consistent with those observed by Vogt et al (1987). Higher gelatin concentration was needed to block the surface more effectively. The results also agreed with the previous reports (Vogt et al 1987) which indicated that gelatin was a weak blocker. However good results were said to have been obtained in some other systems

(Saravis, 1984). Casein and milk have been shown to give much better results than other blocking agents for certain proteins (Kenna et al., 1985) and haptens (Bjercke et al., 1986). Since non-fat dry milk dissolved easily and is inexpensive, it offers certain advantages over purified casein. However it is a complex mixture containing substances that may interfere with certain assays and mask other solid-phase proteins, causing the complete lose of immunoreactivity of antigens bound to the solid phase (Spinola and Cannon, 1985). Fish skin gelatin is an excellent blocker for nitrocellulose Western blots (Saravis, 1984), and appears to be the best of the gelatin preparations tested. It does not solidify (even at high concentration under refrigeration) and is readily available without the need for further processing.

6.5 Conclusion

The optimal blocking agent for a particular assay must be determined by empirical testing. The effectiveness of a blocker in reducing non-specific binding depends on

1. The binding characteristics of the polymer surface used as a solid support and
2. The avidity of binding of the specific proteins and the blocking effect of the added proteins

It was found that on WNR as a solid phase, NEO and NBCS were effective blockers. Both were effective in covering the substrate. However high adsorption of the blockers were also accompanied by high desorption of immobilised proteins (antibodies and binder) which would reduce specific binding. Therefore, two factors must be considered in the choice of suitable blockers : the adsorption of blockers and desorption of immobilised antibody-antigen protein. Gelatin which can cover the unbound surface moderately and does not result in high desorption was considered as a good blocker for WNR surface.

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