Biochimica et Biophysica Acta, 612 (1980) 40—49

BBA 68933

CONJUGATION OF ENZYMES TO IMMUNOGLOBULINS USING DIMALEIMIDES

PETER D. WESTON [a], JOHN A. DEVRIES [a] and ROGER WRIGGLESWORTH [b]

[a] *Department of Reagents and* [b] *Department of Chemistry, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS (U.K.)*

(Received August 30th, 1979)

Key words: Enzyme conjugation; Immunoglobulin; Dimaleimide

Summary

A method is described for coupling enzymes to immunoglobulins using sulphydryl derivatives of the proteins and a dimaleimide which is relatively water-soluble. Parameters affecting the performance of the conjugates have been examined including level of sulphydryl incorporation, ratio of enzyme/immunoglobulin and nature of dimaleimide used. Peroxidase-immunoglobulin conjugates made by the dimaleimide method have been compared with those made by the periodate oxidation method and found to have a superior performance. Immunoglobulin has been linked to peroxidase (horseradish peroxidase, EC 1.11.1.7), glucose oxidase from *Aspergillus niger*, (EC 1.1.3.4), penicillinase from *Bacillus cereus* β-lactamase I (EC 3.5.2.6), and β-galactosidase from *Escherichia coli* (EC 3.2.1.23).

Introduction

Our work in the field of enzyme immunoassay led us to examine the existing techniques for coupling enzyme to antibody (for reviews see Refs. 1—3). The glutaraldehyde methods of Avrameas and Ternynck [4,5] have been widely used to make conjugates for enzyme immunoassay linking a variety of enzymes including peroxidase, alkaline phosphatase and glucose oxidase to antibody or antigen [6]. Nakane and Kawaoi [7] have described a method of linking peroxidase to antibody by periodate oxidation of the carbohydrate moiety of the enzyme; conjugates made in this way have found some application in enzyme immunoassay [8,9]. More recent methods of protein-protein conjugation described by Carlsson et al. [10], Rector et al. [11] and King et al. [12] have not yet been evaluated in immunoassay systems.

Dimaleimides are known as reagents which react rapidly under mild conditions with protein sulphydryl groups [13], and a useful linking method has been developed by Kato et al. [14] using *N,N'-o*-phenylenedimaleimide to couple β-galactosidase to reduced pepsin-digested antibody; the dimaleimide links through the sulphydryl groups which occur naturally in the enzyme and those which have been created by reduction of the $(Fab')_2$ fragment of antibody. Kato et al. [15] have also introduced sulphydryl groups into insulin using the reagent *S*-acetylmercapto succinic anhydride and coupled the derivatised insulin to β-galactosidase. Several enzyme immunoassays using dimaleimide linked conjugates have now been described by Ishikawa and Kato [16].

We wished to prepare enzyme-antibody conjugates for enzyme immunoassay which retained high levels of both enzyme and antibody activity. We set out to extend the approach of Kato's group to new enzymes besides β-galactosidase, to avoid the steps of pepsin digestion and reduction of antibody, and to try the effect of different dimaleimides on the conjugation method.

Materials and Methods

Glucose oxidase (Grade II), peroxidase (Grade II), and β-galactosidase were obtained from Boehringer, and penicillinase from Wellcome Reagents; *N,N'-o*- and *-p*-phenylenedimaleimide, 2,2'- and 4,4'-dithiodipyridine were from Aldrich, hydroxylamine-HCl and 1-fluoro-2,4-dinitrobenzene were from Koch-Light, *S*-acetylmercapto succinic anhydride and penicillin G were from Sigma. All other chemicals were from BDH and of 'Analar' grade whenever available. Sheep anti-human IgG serum was obtained from Wellcome Reagents and the immunoglobulins purified by a rivanol-$(NH_4)_2SO_4$ method, essentially as described by Heide and Schwick [17].

Introduction of protected SH groups

S-Acetylmercapto succinic anhydride was used to introduce protected SH groups into enzymes and immunoglobulin preparations. The method of Klotz and Heiney [18] was extensively changed to the following: before derivatisation, 10—30 mg/ml proteins were dialysed against 0.15 M NaCl and cooled to 0°C. *S*-Acetylmercapto succinic anhydride was dissolved (at up to 40 mg/ml) in dry, redistilled dimethylformamide, stored over Fisher Molecular Sieve, Type 4A. The pH was controlled by the addition of ice-cold 1 N NaOH, using a pH stat Radiometer Titrator set at pH 8.0 during the dropwise, slow addition of the solution of *S*-acetylmercapto succinic anhydride to the stirred ice-cold protein solution. The protein derivative was then dialysed against 0.15 M NaCl with repeated changes of the dialysis liquid and finally stored frozen at −20°C.

Analysis for SH groups

This was done using the method of Grassetti and Murray [19] using 2,2'-dithiodipyridine, or 4,4'-dithiopyridine when extra sensitivity was needed. Before SH analysis the protecting acetyl group was removed from the *S*-acetylmercapto succinic anhydride derivatised proteins by treatment with hydroxylamine for 5 min at pH 7.0 at a final concentration of 0.1 M as described by Klotz and Heiney [18].

Dimaleimides

N,N'-o- and *p*-Phenylenedimaleimide were used without further purification *N,N'*-(oxydimethylene)dimaleimide was synthesised by the method of Tawney et al. [20]; it was a pale yellow solid, melting point 125°C; infrared and NMR spectra supported the structural assignment given in Scheme II.

The maximum solubility of dimaleimide was investigated under the conditions chosen for the derivatisation which were 25% acetone/NaCl (v/v) at room temperature; an increasing level of dimaleimide was dissolved in acetone; and 1 vol. dimaleimide solution was added to 3 vols. protein solution.

Coupling method

The *S*-acetylmercapto succinic anhydride derivatised peroxidase at a concentration between 5 and 30 mg/ml was stirred at room temperature and 1 M hydroxylamine (1.75 g $NH_2OH \cdot HCl$ and 7.1 g anhydrous Na_2HPO_4 made to 25 ml with water) at pH 7.0 was added dropwise (1 vol. per 10 vols. protein solution). The mixture was allowed to stand for 5 min then a solution of dimaleimide (freshly made in acetone) was slowly added, with stirring, to give a final acetone concentration of 25% (v/v). The dimaleimide used was *N,N'*-(oxydimethylene)dimaleimide at 10 mg/ml in acetone, except where otherwise stated. After 10 min standing, the mixture was dialysed against ice-cold 0.1 M phosphate buffer (pH 6.25) overnight, or separated immediately using a column of Sephadex G-25 (medium grade), equilibrated with the same buffer at room temperature. A bed of 1.5 × 19 cm effectively separated a 6-ml sample. The concentration of activated enzyme in the pool of peak fractions from the Sephadex G-25 column or from within the dialysis bag was measured using the absorbance at 280 nm. The extinction for 1 mg/ml solution was taken to be 1.4 for immunoglobulin, 0.86 for peroxidase, 0.79 for glucose oxidase and 1.0 for penicillinase. The *S*-acetylmercapto succinic anhydride derivatised immunoglobulin to be coupled was adjusted to a suitable concentration with 0.15 M NaCl; all peroxidase conjugates were made with an enzyme/immunoglobulin ratio of 1 : 2 (w/w) except in the experiment where the ratio of peroxidase to immunoglobulin was deliberately varied. *S*-Acetylmercapto succinic anhydride derivatised immunoglobulin was treated with hydroxylamine for 5 min as above to deprotect —SH groups, the dimaleimide treated enzyme was added and coupling was allowed to proceed overnight at room temperature. To terminate the coupling, neutralised *N*-acetylcysteine solution (10 mg/ml, 1 : 10 (v/v) solution/conjugate) was added, then after 5 min standing, a solution of 100 mg/ml bovine serum albumin, 100 mg/ml mannitol, 10 mg/ml thiomersal was added (1 : 10 v/v solution/conjugate) as preservative.

Antibody activity

This was measured by the method of single radial immunodiffusion incorporating the immunoglobulin to be tested into agarose gel, essentially as described by Becker [21]; the temperature of mixing was altered to 56°C. The final concentration of immunoglobulin under test was 2.5 mg/ml and of agarose was 1%; 2.0 mg/ml human IgG was inserted into wells cut in the agarose and the diameters of the diffusion rings were read after 24 h at room temperature.

Assay of enzymes

In solution these were measured at 37°C using a Gilford Stasar III spectrophotometer equipped with an automatic timed print out. Enzyme-immunoglobulin conjugates were diluted before assay to about 5 μg enzyme/ml using 0.15 M NaCl solution containing 1 mg/ml bovine serum albumin. The assays for glucose oxidase and peroxidase used the 4-amino phenazone/phenol reagents described by Barham and Trinder [22] and, for penicillinase, the starch-iodine decolourisation reaction originated by Novick [23].

Assessment of enzyme-antibody conjugates in microtitre plates

Multiwell plates with flat bottomed wells made in polystyrene by C.A. Greiner (Nürtingen) were coated with a 5 μg/ml solution of protein in 0.05 M $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) for 3 h at 37°C, then overnight at 4°C. The plates were washed with 0.05% Tween-20 in 0.15 M NaCl and stored at 4°C until use. Dilutions of conjugates were made in 0.05% Tween-20 in 0.15 M NaCl with 5 mg/ml bovine serum albumin and 200 μl filled into wells in the plate. Plates were covered and incubated at 37°C for 30 min then washed with 0.05% Tween-20 in 0.15 M NaCl. Substrate solutions (200 μl per well) were applied using a Micro-Compupet (General Diagnostics, N.J.). The substrate for peroxidase was made according to Ruitenberg et al. [24]; for glucose oxidase the 5-aminosalicyclic acid solution was made up as for peroxidase then (2 g) glucose and (5 mg) peroxidase added and the volume made up to 100 ml. For penicillinase, the starch-iodine substrate was made up as described by Novick [23].

Results and Discussion

As a preliminary to coupling, protected SH groups were introducted into both enzyme and immunoglobulin by use of the reagent *S*-acetylmercapto succinic anhydride. This procedure did not affect the solubility of any of the enzymes examined, peroxidase, glucose oxidase and penicillinase, nor of immunoglobulins prepared from rabbit, horse and sheep sera. Enzyme activity was retained for both peroxidase and glucose oxidase up to the highest levels of *S*-acetylmercapto succinic anhydride offered, however, penicillinase showed some loss. Sheep anti-(human IgG) immunoglobulin at the two highest levels of *S*-acetylmercapto succinic anhydride lost antibody activity, but lower levels left its activity unaltered (Table I).

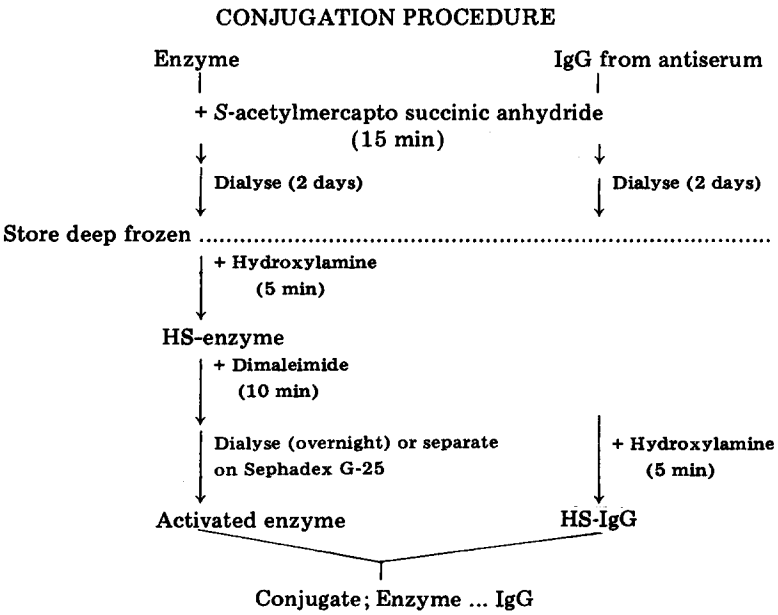
The coupling procedure is shown diagramatically in Scheme I. Prior to treatment with dimaleimide, acetylated SH groups were deprotected by treatment of the *S*-acetylmercapto succinic anhydride derivatised enzyme with aqueous hydroxylamine for 5 min. The dimaleimides used as linking reagents (Scheme II) were soluble in acetone and of the three examined it was found that *N,N'*-(oxydimethylene)dimaleimide was the most soluble (Table II). The greater solubility of the *N,N'*-(oxydimethylene)dimaleimide gives the advantage of a large molar excess of maleimide over SH-enzyme reducing the probability of self polymerisation of the SH-enzyme. Analysis after 10 min reaction of SH-enzyme with *N,N'*-(oxydimethylene)dimaleimide showed that there were no SH groups measurable (Table II) suggesting that the rate of reaction of the

TABLE I

ANALYSIS OF THÉ — SH GROUPS INCORPORATED INTO DIFFERENT PROTEINS AND OF THE BIOLOGICAL ACTIVITY RETAINED

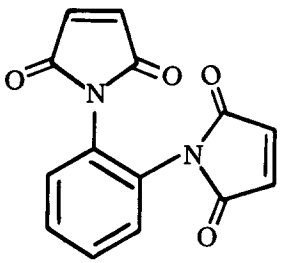
For the purposes of calculation the different proteins were taken to be pure with molecular weights as follows: sheep immunoglobulin, 160 000; peroxidase, 40 000; glucose oxidase, 150 000; penicillinase, 28 000.

Protein	mol reagent offered/ mol protein	mol SH found/ mol protein	% Activity retained in derivatised protein
Peroxidase	Control	0.2	100 by definition
	7.5	0.5	100
	15	1.3	100
	30	3.1	100
	60	6.8	100
	120	9.2	100
Glucose oxidase	Control	0.2	100 by definition
	15	1.1	100
	30	1.6	100
	60	4.0	100
	120	6.5	100
	240	11.4	100
Sheep immunoglobulin	7.5	4.5	100
	15	8.1	100
	30	10.7	100
	60	22.5	0
	120	28.5	0
Penicillinase	42	3.9	74

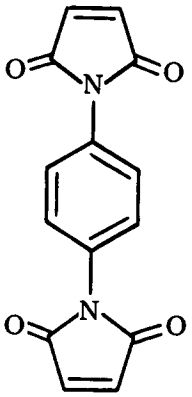


Scheme I.

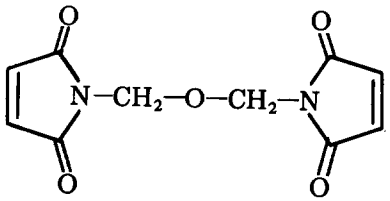
SCHEME 2



N,N'-o-Phenylenedimaleimide



N,N'-p-Phenylenedimaleimide



N,N'-(oxydimethylene)dimaleimide

dimaleimide with SH was rapid and complete; the phenylene dimaleimides gave less efficient removal of SH groups under the same conditions (Table II), the para-substituted compound being the least effective. Enzyme was freed from excess dimaleimide either by Sephadex G-25 separation or by dialysis against

TABLE II

COMPARISON OF DIFFERENT DIMALEIMIDES

An acetone solution of each dimaleimide at the maximum concentration found soluble (1 volume) was mixed with a solution of peroxidase-SH with 8.1 mol SH/mol peroxidase (10 mg/ml, 3 volumes). After standing 10 min the mixture was applied to G-25 Sephadex; the peak fraction of eluted proteins was analysed for SH content.

Dimaleimide	Maximum concentration found soluble (mg/ml)	SH groups measurable after 10 min (as % of original)	Dimaleimide/SH ratio offered (mol/mol)
N,N'-o-Phenylenedimaleimide	4	14	1.2
N,N'-(Oxydimethylene)dimaleimide	20	0	27.9
N,N'-p-Phenylenedimaleimide	0.6	100	0.4

pH 6.25 buffer, and was then ready for coupling to the immunoglobulin. The pH of 6.25 was chosen in view of the known stability and reactivity with SH of maleimide under these conditions [25]. *S*-Acetylmercapto succinic anhydride derivatised immunoglobulin was treated with hydroxylamine in order to deprotect the SH groups and then the dimaleimide activated enzyme was added. The same ratio of peroxidase to immunoglobulin was used for all conjugation mixtures. Coupling was allowed to proceed overnight and the reaction mixture was then assayed for retention of enzyme and antibody activity; the results for both peroxidase and immunoglobulin with differing degrees of SH incorporation are shown in Table III. Activities retained using the coupling technique of Nakane and Kawaoi [7] are recorded for comparison. The dimaleimide method appears to give higher retention of both enzyme and antibody activity giving it a potential advantage over the Nakane and Kawaoi [7] technique, providing that comparable levels of enzyme are attached to immunoglobulin in the two methods.

The performance of the enzyme:anti-human IgG conjugates was measured by adding dilutions of the conjugation mixture to plastic multiwell plates precoated with human IgG. After incubation to allow binding, the plates were

TABLE III

COMPARISON OF PEROXIDASE AND ANTIBODY ACTIVITIES RETAINED IN THE CONJUGATION MIXTURE FOLLOWING DIMALEIMIDE AND PERIODATE LINKAGE OF PEROXIDASE TO IMMUNOGLOBULIN

All these conjugates were made with a ratio of peroxidase/immunoglobulin of 1 : 2 (w/w) for both methods of linkage. Activities recorded in the table are the average of assays done on three separate occasions on the soluble reaction mixtures.

(A) Dimaleimide linking method

mol SH/mol peroxidase	mol SH/mol immunoglobulin	Peroxidase activity retained (%)	Anti-Ig acitivity retained (%)
4.7	8.1	85	84
9.1	10.7	79	93
1.3	10.7	100	92
0.5	10.7	100	78
1.3 *	2.0	100	100
6.8 *	2.0	100	100
1.3 *	11.2	100	100
6.8 *	11.2	90	100

(B) Periodate linking method of Nakane and Kawaoi [7]

Concentration of periodate (M)	Reagent for blocking formyl groups	Peroxidase activity retained (%)	Anti-Ig activity retained (%)
0.08	Sodium borohydride	52	41
0.08	Sodium borohydride	61	<25
0.08	Ethanolamine **	67	80
0.04	Sodium borohydride	69	<25
0.04	Ethanolamine *	77	70

* Reverse method of making; activating immunoglobulin with dimaleimide then addding SH-enzyme.
** Ethanolamine blocking was suggested by Barbour [28].

washed and enzyme substrate applied. Conjugates made by the dimaleimide linking method using *N,N'*-(oxydimethylene) dimaleimide were found to compare favourably with those made by the method of Nakane and Kawaoi [7] giving at least twice the activity.

As a control to examine whether dimaleimide could give any linkage of native proteins in the absence of SH groups, glucose oxidase and immunoglobulin which had not been treated with *S*-acetylmercapto succinic anhydride were carried through the coupling procedure. When the conjugation mixture was tested for performance on a multiwell plate coated with human IgG, it was found to be completely inactive; this suggests that the dimaleimide can only act in the presence of proteins with free SH groups.

It was interesting to find that the maleimide groups attached to activated SH-peroxidase remained capable of coupling with undiminished activity to SH-immunoglobulin after 5 days storage at 4°C in pH 6.25 buffer; storage at room temperature overnight led to a slight loss of coupling activity. The rate of reaction of activated enzyme with antibody was found to be fast; a conjugation mixture was diluted 1000-fold with a buffer containing cysteine at measured times and the diluted material was assessed for performance on a multiwell plate. After 1 min there had been substantial coupling and the reaction was complete at 30 min.

Using the coated multiwell plate as a method of assessment, a variety of parameters affecting the conjugation procedure were examined. All the conjugates of enzyme with immunoglobulin discussed in this paper were prepared at least twice and all were then analysed on at least two separate occasions. It was found that higher levels of *S*-acetylmercapto succinic anhydride incorporation into both peroxidase and glucose oxidase gave conjugates with higher titres; however preliminary results have suggested that those made with lower levels of *S*-acetylmercapto succinic anhydride incorporation give better discrimination between positive and negative sera in enzyme linked immunoadsorbent assays.

Conjugates made with the three different dimaleimides were compared and it was found that those made with *N,N'*-(oxydimethylene) dimaleimide gave a titre that was superior to those made using *N,N'*-*o*-phenylenedimaleimide as used by Ishikawa and Kato [15]. The ortho substituted phenylenedimaleimide was more soluble (Table II) and gave conjugates with a higher titre than the para substituted compound; a disadvantage of the ortho substituted reagent was the non specific binding observed to a control well coated with sheep immunoglobulin. The analysis for SH groups done whilst comparing the different dimaleimides and shown in Table II suggested that the *p*-phenylenedimaleimide would give no conjugation; it was surprising to find that conjugation had indeed occurred.

Conjugates could be made in a reverse manner by treated SH-immunoglobulin with dimaleimide, removing excess dimaleimide, then adding SH-enzyme. Conjugates made in this way showed good performance characteristics in multiwell plates with titres comparable to conjugates made normally. This reverse method is useful where the enzyme already contains SH groups and gave successful conjugation of immunoglobulin to β-galactosidase; this enzyme contains free sulphydryl groups which are not essential for its enzymic

TABLE IV

PER CENT PEROXIDASE ACTIVITY CHANGED TO HIGHER MOLECULAR WEIGHT FOLLOWING CONJUGATION TO IMMUNOGLOBULIN

Peroxidase-SH with 15 SH/mole was activated with *N,N'*-9oxydimethylene)dimaleimide and mixed with immunoglobulin-SH with 11 SH/mole in the proportions shown. Each of the conjugation mixtures (1 ml) were successively applied to Sepharose CL-6B column (Bed 1.6 cm X 77 cm) equilibrated with saline (0.15 M) contianing thiomersal (0.01% w/v) and eluted at a flow rate of 27 ml/hour. Fractions (2.4 ml) were collected and pools of fractions 20—37 and fractions 38—54 were assayed for peroxidase activity.

Peroxidase/Ig ratio (w/w)	Peroxidase activity in high molecular weight pool (%)
1 : 4	61
1 : 2	70
1 : 1	62
2 : 1	44
Control: native peroxidase not activated	0
Control: peroxidase-SH dimaleimide activated and coupled to cysteine	3

activity [26]. One SH-immunoglobulin preparation was activated with dimaleimide and separate aliquots were successfully coupled at the same time to SH-peroxidase, SH-glucose oxidase and SH-penicillinase.

When dimaleimide-activated peroxidase was offered at an increasing level to immunoglobulin the performance of the conjugates improved; maximum utilisation of peroxidase was found using a peroxidase/immunoglobulin ratio of 1 : 2 (w/w) (Table IV).

When enzyme-immunoglobulin conjugates were freeze-dried the solids were difficult to reconstitute. Addition of 10 mg/ml mannitol before freeze-drying gave an improvement and when 10 mg/ml bovine serum albumin was also included the freeze-dried solids reconstituted rapidly to give a clear solution. There was no loss of conjugate activity due to freeze-drying and the dried materials were stable after 3 months storage at 4°C.

The results in this paper show that we have developed a method for coupling peroxidase to immunoglobulin which gives conjugates with high activity and good stability retaining a large proportion of original enzyme and antibody activity. Our preliminary results with glucose oxidase, penicillinase and β-galactosidase suggest that the method can be extended to other enzymes providing that SH groups can be introduced into enzyme without loss of activity. The aliphatic coupling reagent employed, *N,N'*-(oxydimethylene)dimaleimide, offers practical advantages over the aromatic dimaleimides used by earlier workers. The method provides the basis for coupling any two biologically active proteins or other molecules containing sterically accessible amino groups providing that their derivatisation can be achieved without loss of biological activity.

References

1 Kennedy, J.H., Kricka, L.J. and Wilding, P. (1976) Clin. Chim. Acta 70, 1—31
2 Schuurs, A.H.W.M. and Van Weemen, B.K. (1977) Clin. Chim. Acta 81, 1—40

3 Avrameas, S., Ternynck, T., and Guesdon, J.L. (1978) Scand. J. Immunol. 8, Suppl. 7, 7—23
4 Avrameas, S. (1969) Immunochemistry 6, 43—52
5 Avrameas, S. and Ternynck, T. (1971) Immunochemistry 8, 1175—1179
6 Voller, A., Bidwell, D.E. and Bartlett, a. (1976) Bull. World Health Organ. 53, 55—65
7 Nakane, P.K. and Kawaoi, A. (1974) J. Histochem. Cytochem. 22, 1084—1091
8 Wei, R., Knight, G.J., Zimmerman, D.H. and Bond, H.E. (1977) Clin. Chem. 23, 813
9 Hevey, R., Bonacker, L.H. and Sparacio, R. (1976) in First International Symposium on Immunoenzymatic Techniques, INSERM Symposium No. 2 (Feldmann, G., ed.), pp. 191—198, North-Holland, Amsterdam
10 Carlsson, J., Drevin, H. and Axen, R., (1978) Biochem. J. 173, 723—737
11 Rector, E.S., Schwenk, R.J., Tse, K.S. and Sehon, A.H., (1978) J. Immunol. Methods 24, 321—336
12 King, T.P., Li, Y., Kochoumian, L., (1978) Biochemistry 17, 1499—1506
13 Wold, F. (1972) Methods Enzymol. 25, 629—632
14 Kato, K., Fukui, H., Hamaguchi, Y. and Ishikawa, E., (1976) J. Immunol. 116, 1554—1560
15 Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E., (1975) J. Biochem. 78, 235—237
16 Ishiskawa, E. and Kato, K. (1978) Scand. J. Immunol. 8, Suppl. 7, 43—55
17 Heide, K. and Schwick, H.G., (1973) in Handbook of Experimental Immunology (Weir, D.M., ed.), 2nd edn., pp. 61—6.11, Blackwell Scientific Publications, Oxford
18 Klotz, I.M. and Heiney, R.E. (1962) Arch. Biochem. Biophys. 96, 605—612
19 Grassetti, D.R. and Murray, J.F. (1967) Arch. Biochem. Biophys. 119, 41—49
20 Tawney, P.O., Snyder, R.H., Conger, R.P., Leibbrand, K.A., Stiteler, C.H. and Williams, A.R. (1961) J. Org. Chem. 26, 15—21
21 Becker, W. (1969) Immunochemistry 6, 539—546
22 Barham, D. and Trinder, P. (1972) Analyst 97, 142—145
23 Novick, R.P. (1962) Biochem. J. 83, 236—240
24 Ruitenberg, E.J., Ljungstrom, I., Steerenberg, P.A. and Buys, J. (1975) Ann. N.Y. Acad. Sci. 254, 296—303
25 Gregory, J.D. (1955) J.Am. Chem. Soc. 77, 3922—3923
26 Craven, G.R., Steers, E. and Anfinsen, C.B. (1965) J. Biol. Chem. 240, 2468—2477
27 Barbour, H.M. (1976) J. Immunol. Methods 11, 15—23