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AFFINITY PURIFICATION AND PROPERTIES OF PORCINE BRAIN ALDOSE REDUCTASE

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Summary

Aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21) has been purified 1500-fold from porcine brain in a four-step procedure employing Blue-Sepharose 6B affinity chromatography. The purified enzyme was shown to be apparently homogeneous by polyacrylamide gel electrophoresis. The enzyme is a single chain polypeptide of molecular weight 40 000, pH optimum 5.0, K^{app}_{xylose} 4 mM; K^{app}_{NADPH} 3 μM. The relative substrate activities, activation with sulfate ion, and limited oxidative and NADH-related reductive activities confirm the classification of this enzyme as aldolase reductase. The activity of the reductase with *p*-nitrobenzaldehyde and 3-indolacetaldehyde and the similarity of its physical properties with the 'low K_m' aldehyde reductase of porcine brain previously reported indicates that these enzymes may be identical.

Aldose reductase (alditol:$NADP^+$ 1-oxidoreductase, EC 1.1.1.21) catalyzes the reduction of a variety of sugars to the corresponding polyol in an $NADP^+$-linked reaction:

$$\text{Aldose} + \text{NADPH} + H^+ \rightleftharpoons \text{Polyol} + NADP^+$$

While the enzyme has been identified in a variety of mammalian [1—8] and microbial [9—11] sources, its metabolic significance in many of these cases is not yet fully understood. However, as recently reviewed [12], there is consider-

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able interest in this enzyme because of its postulated role in the development of many of the secondary effects of diabetes. Kinoshita [13] has shown a clear relationship between the biochemistry of aldose reductase and its physiological consequences in his studies of sugar-cataract formation in mammalian lens.

Although lens tissue is known to contain aldose reductase as the only NADPH dependent aldehyde reducing enzyme [2,4,14], brain tissue contains, in addition, the enzyme variously referred to as L-hexonate dehydrogenase (EC 1.1.1.19) or D-glucuronate reductase [3,4,6,14]. Whether the enzymes catalyzing the NADPH dependent reduction of aldehydes derived by oxidative deamination of biogenic amines in the brain [15,16] are different from these enzymes remains to be established.

In this paper we report the physical and kinetic characteristics of an aldose reductase purified from porcine brain by affinity gel chromatography.

Materials

DL-glyceraldehyde, xylose, nucleotide cofactors and porcine brain acetone powder were obtained from Sigma Chemical Co. (St. Louis, MO 63178). Chromatographic support and associated media were purchased from Whatman (Clifton, NJ 07014, DE-52 ion exchange cellulose), Pharmacia (Piscataway, NJ 08854, Sephadex gels and Blue Sepharose 6B) and Bio-Rad Laboratories (Richmond, CA 94804, electrophoresis gels and stains). Coomassie Brilliant Blue G-250 was purchased from Serva (Heidelberg, F.R.G.). All other materials were of Analytical Reagent grade.

Methods

Protein determination. Static measurement of protein was performed using the methods of Lowry et al. [17] or Murphy and Kies [18]. Column eluate was monitored by determining the absorbance at 280 nm. The protein levels of the affinity column gradient eluates were determined after dialysis of each fraction to avoid the interference arising from the presence of nucleotide cofactor in the eluant.

Enzyme measurement. Enzyme activity was determined routinely at 30°C by following the decrease in absorbance at 340 nm on a Gilford 300-N spectrophotometer equipped with a Model 4006 data lister, or, when large numbers of column eluates were to be tested, on an Abbott ABA-100 bichromatic analyzer (fast kinetic mode; 1 : 51, sample : reagent ratio). Each reaction mixture contained 60 mM phosphate buffer (pH 6.2), 136 μM NADPH, 0.90 mM DL-glyceraldehyde. The reaction was initiated by the addition of enzyme to a total final volume of 1.10 ml. One unit of activity is defined as that amount of enzyme which catalyzes the oxidation of 1 μmol NADPH h^{-1} with DL-glyceraldehyde as substrate. D-Xylose (90 mM) was the substrate used in the determination of the pH optimum. Relative enzyme activities in the various fractions were determined in the same system containing, alternately, 0.9 mM DL-glyceraldehyde, 90 mM D-xylose, 9 mM sodium D-glucuronate and 0.23 mM *p*-nitrobenzaldehyde.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was

performed as described by David [19]. Gels with 4, 7.5 and 12% monomer concentration were run in a similar manner with Tris-glycine buffer at pH 8.9. Protein was stained using Coomassie Brilliant Blue R-250 [20] or G-250 in $HClO_4$ [21]. Staining for enzymic oxidative activity was done in phosphate or bicarbonate buffers over the pH range 7.0—9.8, using glycerol, sorbitol, or erythritol as substrates and NAD^+ or $NADP^+$ as cofactor at 37°C [22]. The reduced cofactor was coupled to phenazine methosulfate and nitro blue tetrazolium. The reverse of this approach was used in staining for aldose reduction. Gels were incubated at 37°C for 1 h in the routine assay mixture. The gels were washed, then incubated in 5 mM phosphate buffer (pH 7.4) containing 0.1 mg/ml nitro blue tetrazolium and 0.02 mg/ml phenazine methosulfate. Reducing activity is evident as a clear band on an otherwise blue gel.

Molecular weight determinations in the presence of sodium dodecyl sulfate (SDS) were performed by the method of Weber and Osborn [23] using human serum albumin (68 000), catalase (60 000), ovalbumin (43 000), yeast alcohol dehydrogenase (37 000) and trypsin (23 000) as standards.

Gel permeation chromatography. Molecular weight determinations on Sephadex G-100 were carried out as previously described [24] with aldolase (158 000), human hemoglobin (65 000), hen albumin (45 000), chymotrypsinogen A (25 000) and cytochrome c (12 500) as standards.

Purification of the enzyme. All steps were performed at 4°C in the presence of 1 mM dithiothreitol.

(1) *Crude extract.* Pig brain acetone powder was suspended in $5 \cdot 10^{-3}$ M

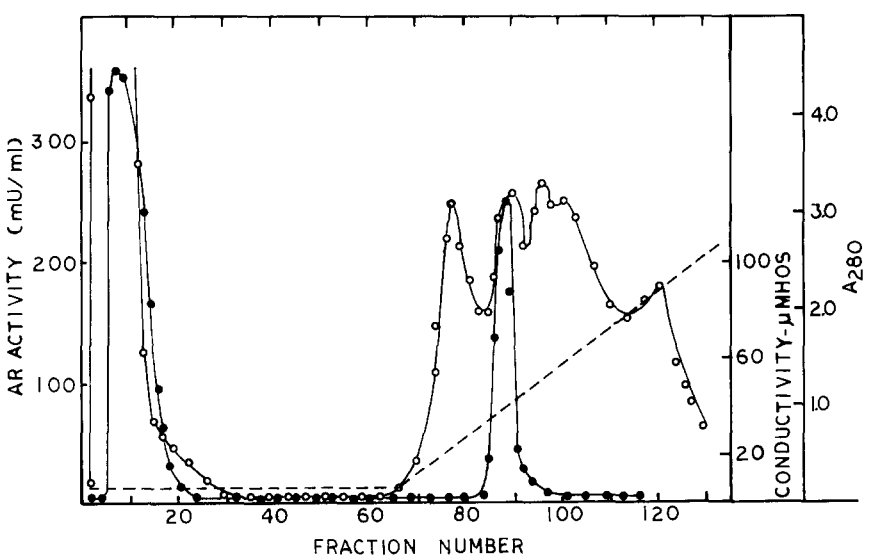

Fig. 1. DEAE-cellulose ion-exchange chromatography. Fraction 2 was applied to the 2.6 × 30 cm column previously equilibrated at 4°C with 5 mM phosphate buffer (pH 7.4), 1 mM in dithiothreitol. Elution with the same buffer was continued until the unbound protein (fron peak, A_{280nm}) was completely removed. Gradient (50 to 150 mM) elution in the buffer was then initiated. Protein (○———○), enzyme (●———●) and conductivity (- - - - -) measurements were carried out on each or alternate 5 ml, fractions (4X concentration, PM-10 Amicon ultrafilter membrane). A unit of activity as measured on the ABA-100 is expressed here as μmol NADPH min^{-1}.

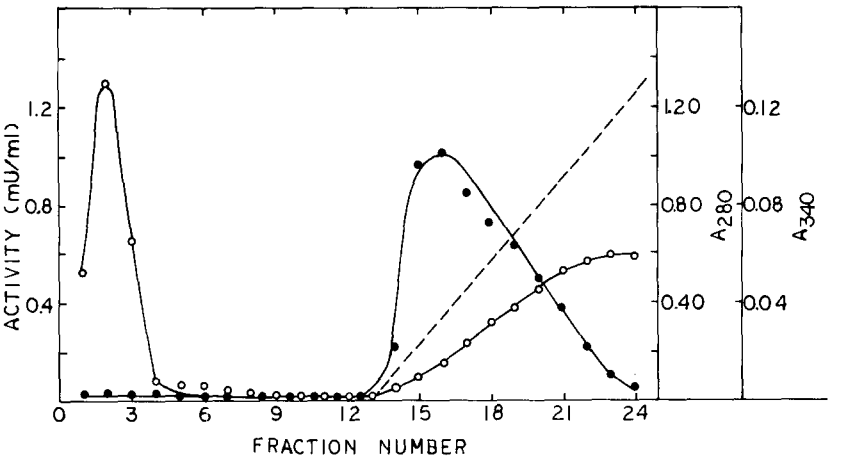

Fig. 2. Affinity chromatography on Blue Sepharose 6B. An aliquot (10 mg protein, fraction 3) was applied to the gel column (0.7 X 7 cm) in the equilibrating and eluting 0.01 M phosphate buffer (pH 6.2). Protein (○———○) and enzyme (●———●) activity (μmol/h) were measured. The counter-ligand gradient (0—0.2 mM NADPH) was monitored at 340 nm (- - - - -). Protein wash fractions (13 ml) and gradient wash fractions (2.5 ml) were collected at a flow rate of 0.43 ml min^{-1}.

Tris-phosphate buffer (pH 7.4) and extracted using a motor driven Teflon and glass homogenizer. The resulting homogenate was centrifuged at 48 000 X *g* for 30 min. (The same procedure was followed for fresh pig brain using 2 vols. buffer).

(2) *$(NH_4)_2SO_4$ treatment.* The precipitate sedimenting at 35—80% saturation of $(NH_4)_2SO_4$ was redissolved in 0.005 M phosphate buffer, pH 7.4, and dialyzed 3 times (8 h each) against 10 vols of the same buffer.

(3) *DE-52 cellulose chromatography.* The dialyzed enzyme preparation (fraction 2) was applied to a (2.6 X 30 cm) column of DE-52 cellulose previously equilibrated in 5 mM phosphate buffer (pH 7.4). The column was washed with the same buffer until the absorbance at 280 nm was almost zero then developed with a 0.005—0.150 M linear phosphate buffer gradient (pH 7.4) (flow-rate 42 ml/h). The column eluate was directed through an (Amicon) on-line

TABLE I

PURIFICATION SEQUENCE FOR PORCINE BRAIN ALDOSE REDUCTASE

Step	Volume (ml)	Protein (mg)	Activity *		Purification **	
			Total	Specific	Yield (%)	Fold
Crude extract	348	17 530	1287	0.073	100 (100)	1 (1)
$(NH_4)_2SO_4$	167	3 865	746	0.19	58 (58)	3 (3)
Ion-exchange (DE-52)	42	59	244	4.1	19 (47)	57 (143)
Affinity gel (Blue Sepharose 6B)	34	1.0	44	44	3.4 (8)	603 (1517)

* Total activity is expressed as μmol NADPH h^{-1}; specific activity is given as μmol NADPH h^{-1}/mg protein.

** Values in parenthesis represent yields and fold purification corrected for hexonate dehydrogenase.

concentrator fitted with a PM-10 ultrafiltration membrane. The characteristic elution pattern resulting from this treatment is shown in Fig. 1.

(4) *Affinity gel chromatography*. An aliquot from the pool of active fractions from the DEAE-cellulose gradient step (fraction 3), containing about 10 mg protein, was applied to a (0.7 X 7.0 cm) column of Blue-Sepharose 6B (2 μmol dye/ml gel) equilibrated in 0.01 M phosphate buffer, (pH 6.2). When the column wash showed no further evidence of protein being eluted, a linear 0—0.2 mM NADPH gradient in phosphate buffer was initiated. The two 2.5 ml fractions of peak activity emerging with the NADPH-gradient (Fig. 2) were collected and shown to contain purified aldolase reductase. Protein values of the enzyme fractions were determined after dialysis or buffer exchange on Sephadex G-25, since the nucleotide interferes with the routine monitoring of protein at 280 nm.

The purified enzyme (fraction 4) was stored at 4°C if it was to be used immediately. Otherwise 1 mg/ml of bovine serum albumin was added and it was stored frozen at −12°C. A typical purification scheme is shown in Table I.

Results

Purification. Approx. 70% of the total reducing activity with D,L-glyceraldehyde as substrate was due to L-hexonate dehydrogenase, which, as indicated by the relative substrate activities and electrophoretic behavior of fractions 3 and 4, (Fig. 1) is completely separated from aldose reductase in the ion-exchange step. Since the remainder of the activity (30%) is due to aldose reductase, the yield and fold purification (Table I) is shown to express this fact. If the ion-exchange step is omitted from the purification sequence, the resulting yield and specific activity are 11% and 4.8 U/mg, but the relative substrate activities of this modified preparation remain unchanged. Thus the large amounts of contaminating hexonate dehydrogenase appear to be completely removed from the reductase by the $(NH_4)_2SO_4$ and DE-52 ion-exchange chromatography steps, or alternatively, by $(NH_4)_2SO_4$ treatment and affinity gel chromatography.

NADPH (0.2 mM) was found to be the most effective counter ligand for removing the enzyme from the Blue Sepharose 6B. The elutability of the reductase was not affected when 0.05 M NaCl was incorporated into the irrigating buffer. ATP (10 mM), (10 mM) NAD^+ and 1 mM $NADP^+$ were not as effective as the cosubstrate for aldose reduction in eluting the reductase from the column. In fact, ATP and NAD^+ at these concentrations were found in a separate experiment to completely inhibit aldose reductase activity.

These data indicate that the affinity gel behavior of the enzyme can be characterized as biospecific adsorption [25] in which the interaction of the reductase with the Cibacron Blue F3GA ligand is relatively weak. It will be recalled that in this gel the ligand is joined directly to the agarose matrix throught the triazine moiety, and no spacer arm is present.

When fresh frozen porcine brain was used as the source and taken up in the same extracting buffer, comparable yields and activities relative to the equivalent weight of acetone powder were obtained (100 g fresh brain tissue yields approx. 20 g acetone powder). Lower yields resulted if dithiothreitol was

absent in either case, or if the temperature was allowed to rise above about 4°C, particularly in steps 3 and 4.

Stability of the preparation

The purified enzyme gradually lost all activity at 4°C after 5 to 7 days and about 90% of its activity upon rapid freezing in an acetone/solid CO_2 bath or slow cooling to −12°C in a freezer. The addition of 1 mg/ml bovine serum albumin stabilized the preparation so that no activity was lost after 50 days at −12°C or 2 days at 4°C. All characterization studies were carried out on the freshly prepared enzyme prior to the addition of albumin. Kinetic studies were performed on the serum albumin stabilized enzyme after it was established that the addition of albumin did not alter the relative activities or the apparent K_m with xylose as substrate.

Apparent Michaelis constants

The apparent Michaelis constant for xylose (K_{xyl}^{app}) in the presence of NADPH (5—136 μM) was 4.0 ± 0.3 mM. No evidence for xylose inhibition was observed up to a concentration of 2 M. K_{NADPH}^{app} (in 24 mM xylose) was 3.1 ± 0.2 μM. Enzyme inhibition was observed, beginning at 90 μM NADPH, and increased with increasing cofactor concentration. At 136 μM, the reaction was about 15% inhibited, relative to the maximum activity at 60—80 μM.

pH activity profile

The activities of the enzyme in phosphate and MES (2-(*N*-morpholine) ethanesulfonic acid) buffers (I = 0.1) at various pH values relative to the (optimum) activity in MES at pH 5.0 are shown in Fig. 3. Enzyme activity in phosphate buffer at constant concentration (0.060 M) in the pH range 6.2 to 7.9 showed no differences relative to the corresponding phosphate buffers at constant ionic strength. Equivalent activities were found in MES buffer at two

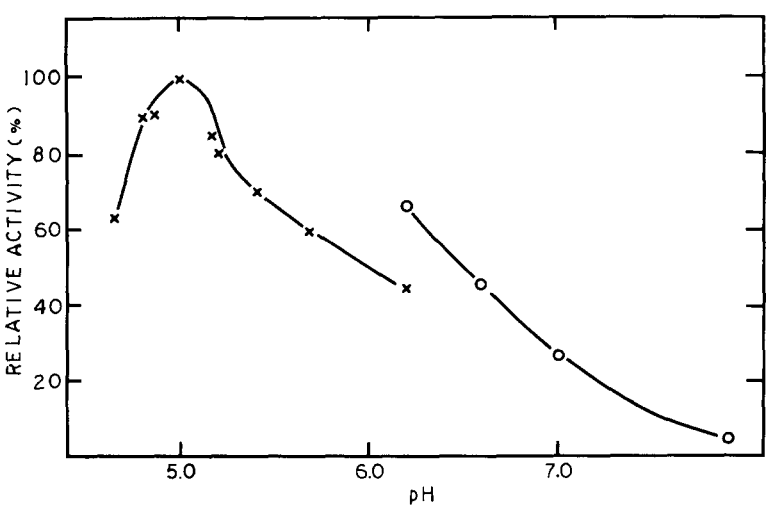

Fig. 3. Influence of pH on the reducing activity of purified aldose reductase. Activities were measured with xylose as substrate. ○———○, Phosphate; MES, X———X. Ionic strengths, 0.1.

different ionic strengths (I = 0.05 and 0.1). The activity in 0.06 M acetate buffer was less than half that in MES buffer in the pH range from 5.2 to 5.8.

When 0.4 M Li_2SO_4 was incorporated into the 0.06 M phosphate buffer, the activity of the reductase was increased by 60% above that recorded for the phosphate buffer in the absence of the lithium salt over the pH range 5.7 to 6.4.

Relative substrate activities

The activities of varous fractions with several aldehyde substrates relative to DL-glyceraldehyde are shown in Table II. The crude and ammonium sulfate fractions showed some variation in relative activity from one preparation to another, reflecting the variation in the fraction of total activity contributed by aldose reductase. Within 1 mM 3-indolacetaldehyde as substrate the activity of fraction 4 was 43% of that with 1 mM DL-glyceraldehyde. If 136 μM NADH was substituted in the assay with either DL-glyceraldehyde or *p*-nitrobenzaldehyde as substrate, only about 3—5% of the activity was found, relative to NADPH, indicating the enzyme is NADPH specific. No reaction with 450 mM glycerol and 115 μM $NADP^+$ or 115 μM NAD^+ was observed for the enzyme in glycine-NaOH buffers from pH 8.6 to 10.4.

The activity of our preparation with DL-glyceraldehyde as substrate was increased about 60% in the presence of 0.4 M Li_2SO_4. Similar activation was observed in the presence of $(NH_4)_2SO_4$, suggesting that the SO_4^{2-} ion is the activating species. However, the relative activities of the other substrates were altered (also noted by Hers, see Ref. 1) as indicated in Table II. As a result of these variations in the relative substrate activity, Li_2SO_4 was not used further in the studies described here.

Electrophoretic and chromatographic behavior

An aliquot of fraction 4 containing 30 μg protein yielded a single band upon electrophoresis and protein staining on each of the various pore size gels.

TABLE II

RELATIVE SUBSTRATE ACTIVITIES

Step/fraction	Substrates *			
	DL-Glyceraldehyde	D-Xylose	D-Glucuronate	*p*-Nitrobenzaldehyde
1 Crude extract	100	70	410	410
2 DE-52 (protein wash)	100	55	650	610
3 DE-52 (gradient, fraction 3)	100	93	58	100
4 Affinity gel				
(i) Before removal of NADPH **	100	94	67	110
(ii) After removal of NADPH	100	91	64	95
(iii) Before removal of NADPH in the presence of 0.4 M Li_2SO_4	100	76	30	81

* Substrate concentrations used were: 0.90 mM DL-glyceraldehyde; 90 mM D-xylose; 9 mM D-glucuronate 0.23 mM *p*-nitrobenzaldehyde; 136 μM NADPH.

** Fraction 4.

Assuming a Coomassie Blue R-250 sensitivity of 20 ng of protein [8], the absence of additional bands indicates that no other protein is present at greater than 0.1% of the concentration of aldose reductase.

When aliquots of fractions 1 through 4 were stained for aldose reducing activity following electrophoresis, a single band corresponding to hexonate dehydrogenase was observed in fractions 1 and 2 and in the protein wash of the DE-52 column. No band corresponding to aldose reductase activity could be observed for the reaction in either direction because of the thermal instability of the enzyme.

A single protein band was also seen on SDS polyacrylamide gels. The molecular weight of the reductase determined by this procedure was 39 000 ± 1600.

The molecular weight of the enzyme as determined from the plot of the calibration curve constructed for Sephadex G-100F was 41 500 ± 3000. Only a single (symmetrical) protein band was observed by column monitoring of the column eluate at 280 nm.

Discussion

The rapid general-ligand affinity gel preparation of porcine brain aldose reductase described in this paper achieves a comparable or improved degree of purity relative to brain tissue preparations from other mammalian species [3,8]. The salt insensitive biospecificity of our preparation and its elutability relative to the Cibacron Blue F3GA affinity ligand with low levels of NADPH as counter-ligand is taken as evidence for the presence of the dinucleotide fold in the enzyme [26]. Gradient or pulse elution with 0.1 mM NADPH, sufficient to fully desorb the enzyme, is consistent with the low value for the apparent Michaelis constant of NADPH.

A summary compilation of the physical properties of several mammalian aldose reductases culled from the literature [2,3,5,6,8,14] follows the pattern; Molecular weight, 30 000—40 000; pH optimum between 5.0—6.7; relative substrate activities, D,L-glyceraldehyde > D-xylose > D-glucuronate; activity with NADH < 20% of activity with NADPH; increased activity in the presence 0.4 M SO_4^{2-}; K^{app}_{NADPH}, 1.2—45 μM; K^{app}_{xylose}, 9—30 mM; negligible reverse reaction. Based on these criteria, the enzyme described in this paper is classed as an aldose reductase. Differences are, however, to be noted. For example, the molecular weight reported for the ox-brain preparation [3] is 56 000. In contrast to the absence of SO_4^{2-} ion activation and the biphasic character of the Lineweaver-Burk plot for D,L-glyceraldehyde reported by Dons and Doughty for the calf brain enzyme [8], we observed pronounced SO_4^{2-} activation and linearity in the Lineweaver-Burk plot with xylose as substrate for the procine brain preparation. We found no evidence for reductase isoenzymes as reported by Gabbay and Cathcart [6].

The similarities and overlapping properties of the NADPH-dependent aldehyde reducing enzymes have been recognized by several groups [15,27,28]. It is to be noted that the pig brain 'low K_m' aldehyde reductase (EC 1.1.1.2) described by Turner and Tipton [16] has a similar molecular weight (36 000), pH optimum (5.0) and K^{app}_{NADPH} (1.2 μM) to those reported here for the pig brain reductase. In addition, our enzyme was found to be active with *p*-nitro-

benzaldehyde and 3-indoleacetaldehyde. The cumulative evidence presented here suggests these two enzymes may be identical.

References

1 Hers, H.G. (1960) Biochim. Biophys. Acta 37, 120—126
2 Hayman, S. and Kinoshita, J.H. (1965) J. Biol. Chem. 240, 877—882
3 Moonsammy, G.I. and Stewart, M.A. (1967) J. Neurochem. 14, 1187—1193
4 Clements, J., R.S., Weaver, J.P. and Winegrad, A.I. (1969) Biochem. Biophys. Res. Commun. 37, 347—353
5 Clements, Jr., R.S. and Winegrad, A.I. (1972) Biochem. Biophys. Res. Commun. 47, 1473—1479
6 Gabbay, K.H. and Cathcart, E.S. (1974) Diabetes 23, 460—467
7 Attwood, M.A. and Doughty, C.C. (1974) Biochim. Biophys. Acta 370, 358—368
8 Dons, R.R. and Doughty, C.C. (1976) Biochim. Biophys. Acta 452, 1—12
9 Scher, B.M. and Horecker, B.L. (1966) Arch. Biochem. Biophys. 116, 117—128
10 Sheys, G.H., Arnold, W.J., Watson, J.A., Hayashi, J.A. and Doughty, C.C. (1971) J. Biol. Chem. 246, 3824—3827
11 Suzuki, T. and Onishi, H. (1975) Agr. Biol. Chem. 39, 2389—2397
12 Gabbay, K.H. (1975) Ann. Rev. Med. 26, 521—536
13 Kinoshita, J.H. (1974) Invest. Opthamol. 13, 713—724
14 Sheaff, C.M. and Doughty, C.C. (1976) J. Biol. Chem. 251, 2696—2702
15 Turner, A.J. and Tipton, K.F. (1972) Eur. J. Biochem. 30, 361—368
16 Turner, A.J. and Tipton, K.F. (1972) Biochem. J. 130, 765—772
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Murphy, J.B. and Kies, M.W. (1960) Biochim. Biophys. Acta 45, 382—384
19 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
20 Meyer, T.S. and Lamberts, B.L. (1965) Biochim. Biophys. Acta 107, 144—145
21 Reisner, A.H., Nemes, P. and Bucholtz, C. (1975) Anal. Biochem. 64, 509—516
22 Smith, I. (1968) Chromatographic and Electrophoretic Techniques, 2nd ed. Vol. 14, pp. 345—351, Interscience, New Jersey
23 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
24 McGuinness, E.T. (1973) J. Chem. Ed. 50, 826—830
25 Barry, S. and O'Carra, P. (1973) Biochem. J. 135, 595—607
26 Thompson, S.T., Cass, K.H. and Stellwagen, E. (1975) Proc. Natl. Acad. Sci. U.S. 72, 669—672
27 Tulsiani, D.R. and Touster, O. (1977) J. Biol. Chem. 252, 2545—2550
28 Bosron, W.F. and Prairie, R.L. (1973) Arch. Biochem. Biophys. 154, 166—172