Biochimica et Biophysica Acta, 377 (1975) 61—70

BBA 67396

COUPLED OPTICAL RATE DETERMINATIONS OF AMINO ACID OXIDASE ACTIVITY

DAVID J. HOLME and DAVID M. GOLDBERG

Department of Chemistry and Biology, Polytechnic, Pond Street, Sheffield S1 1WB and Department of Chemical Pathology, Royal Hospital, West Street, Sheffield S1 3SR (U.K.)

(Received June 17th, 1974)

(Revised manuscript received September 30th, 1974)

Summary

Methods are described in which liberation of ammonia from amino acid substrates by the D- and L-amino acid oxidases may be coupled with the NADH-dependent reductive amination of 2-oxoglutarate catalysed by exogenous glutamate dehydrogenase (L-glutamate: NAD oxidoreductase (deaminating), EC 1.4.1.2). The inhibition of D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) by ADP needed to activate and stabilise glutamate dehydrogenase was relieved by FAD, and the substrate was D-alanine at approximately 6-fold K_m concentration. Neither FAD or FMN were required in the L-amino acid oxidase (L-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) assay; this utilised L-leucine as substrate in a concentration approximately 7-fold the K_m value.

The methods were reasonably sensitive and precise, and a linear relationship between activity and enzyme concentration prevailed up to an absorbance change of 0.050 per min. They have the advantage of being amenable to automation and to employment of fluorescence techniques should greater sensitivity be required.

Introduction

Methods previously employed to determine the activity of amino acid oxidases have included Warburg manometry [1—3]; measurement of ammonia production utilising microkjeldahl determination [1] or colorimetric assay [4]; oxygen polarography [5—7]; various colorimetric procedures [8,9]; and an enzyme-inhibition technique [10]. Such methods are relatively cumbersome, especially if the object is to measure reaction rates with a large throughput of samples. No other technique is so convenient and flexible in this respect as

continuous spectrophotometric monitoring of an enzyme reaction. No general kinetic spectrophotometric assay has yet been presented for the amino acid oxidases. Certain methods depend upon spectral changes developed after completion of the enzyme reaction [11—14]. Two other methods permit continuous monitoring of the reaction [15,16] but are restricted to model substrates rather than to naturally occurring amino acids and have only limited application; moreover the spectral changes occur at wavelengths (250—260 nm) limiting the amount and type of sample that can be tested.

Since ammonia is liberated by action of the enzymes on all amino acid substrates, continuous spectrophotometric methods based upon measurement of ammonia generation have obvious attractions. Such an approach, suggested by previous experience of applying this technique to other enzyme assays [17—20], is developed in the present communication and is based upon the following coupled reactions (GDH: glutamate dehydrogenase):

$$RCHNH_2COOH + O_2 \xrightarrow{\text{oxidase}} RCOCOOH + H_2O_2 + NH_3 \quad (1)$$

$$NH_3 + \text{2-oxoglutarate} + NADH \xrightarrow{GDH} \text{L-glutamate} + NAD^+ \quad (2)$$

The resulting fall in absorbance at 340 nm can be readily monitored.

Materials and Methods

Method A

This was the coupled spectrophotometric assay developed as a consequence of the experiments described in this report. The following reagents were prepared: (1) 0.1 M glycylglycine (Sigma, Cat. No. G 1002), pH 8.3 and 7.5 at 37°C for the D- and L-assays, respectively, adjusted with the Micro Astrup pH Meter (Radiometer, Copenhagen). Other buffers used in certain experiments were Tris, barbitone, and Tricine (*N*-tris-(hydroxymethyl)-methylglycine), all from British Drug Houses (Poole, England). (2) 0.03 M 2-oxoglutarate, monosodium salt (Sigma, Cat. No. K 1875), adjusted to pH 7.0 with NaOH. (3) 2.25 mM NADH, disodium salt (Boehringer Cat. No. 15142 CNAB), freshly prepared. (4) 15 mM ADP, disodium salt (Sigma, Cat. No. A 0127), freshly prepared. (5) 0.3 mM FAD, disodium salt (Sigma, Cat. No. F 6625), freshly prepared. (6) Glutamate dehydrogenase, 10 mg per ml 50% glycerol (v/v), approximate activity 45 units per mg (Boehringer, Cat. No. 15324 EGAH). (7) 0.5 M D-alanine (Sigma, Cat. No. A 7377). (8) 0.1 M L-leucine (Sigma, Cat. No. L 8000).

The method is outlined in Table I and typical assays are shown in Figs 1 and 2. On addition of the sample to the reaction mixture from which substrate has been omitted, there is a rapid fall in absorbance at 340 nm due to oxidation

Fig. 1. Typical trace of spectrophotometric D-amino acid oxidase assay (135 units/liter) on Beckman DBGT with full scale expansion = 1.0 absorbance, chart speed 0.5 inch per min, and back-off approximately 0.30 absorbance. Arrow indicates time of addition of substrate (D-alanine, 0.1 ml of 0.5 M solution). Fall in absorbance before this is due to NH_3 in reagents and sample. No blank reaction is evident with this sample. Subsequent fall is due to enzyme reaction; note initial lag and late fall-off, with middle portion of progress curve being essentially linear.

TABLE I

PROTOCOL FOR COUPLED SPECTROPHOTOMETRIC ASSAYS

Reagent No. (see text)	Volume per assay (ml)	
	D-Amino acid oxidase	L-Amino acid oxidase
Buffer, pH 7.5 (1)	—	2.15
Buffer, pH 8.3 (1)	2.05	—
2-Oxoglutarate (2)	0.20	0.20
NADH (3)	0.20	0.20
ADP (4)	0.10	0.10
FAD (5)	0.10	—
Glutamate dehydrogenase (6)	0.05	0.05
Sample	0.20	0.20
	Equilibrate at 37°C until absorbance stable or decreasing at linear rate; if so, measure this blank reaction	
D-Alanine (7)	0.10	—
L-Leucine (8)	—	0.10
	Determine rate of reaction from linear portion after initial lag period	

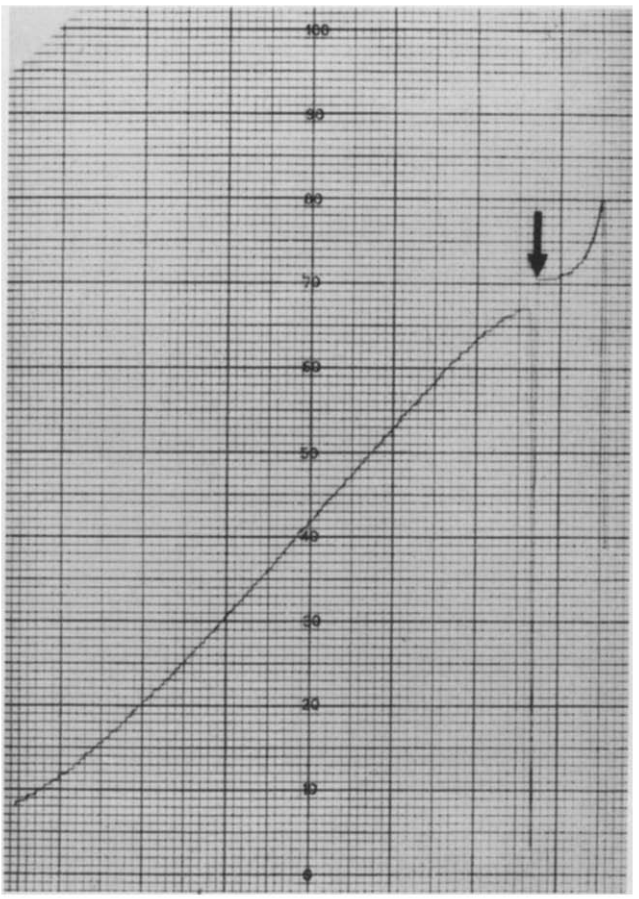

Fig. 1.

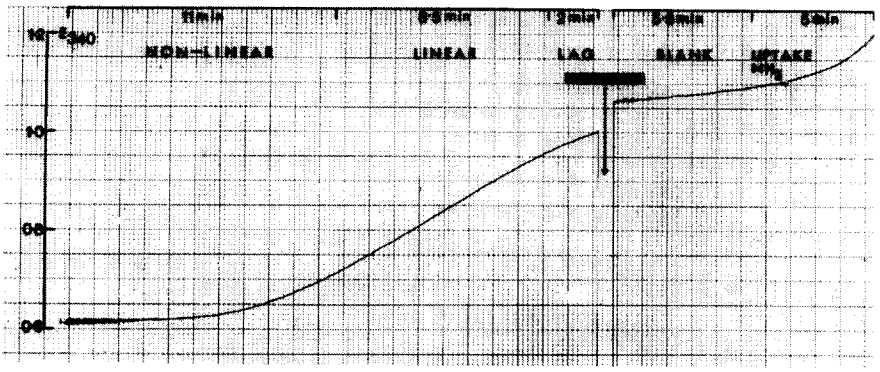

Fig. 2. Typical trace of spectrophotometric L-amino acid oxidase assay using rat kidney supernatant to show stages of assay when using crude enzyme preparation. Arrow indicates addition of substrate (L-leucine, 0.1 ml of 0.1 M solution). The blank was equivalent to 13 units/liter and the linear portion of the test to 77 units/liter; the enzyme activity of the preparation was thus 64 units/liter.

of NADH by ammonia present in sample and reagents. The absorbance may stabilise or continue to fall at a constant low rate depending upon the extent of endogenous ammonia formation by the sample. This represents the blank which should be measured and subtracted from the test. The appropriate substrate is then added to initiate the reaction; after a lag period of 2—4 min, the rate of fall in absorbance is linear until the NADH concentration becomes rate limiting. This is measured and activity as units per liter calculated as:

$$\frac{(\Delta A_{340\,nm} \text{ per min test} - \Delta A_{340\,nm} \text{ per min blank}) \times 1.5 \cdot 10^4}{6.22}$$

In the present work the instrumentation comprised a DBGT Recording Spectrophotometer (Beckman Instruments, Glenrothes, Fife, Scotland) with thermal cuvets maintained at 37°C by an external circulating water bath.

Method B

This measured enzyme activity as the rate of oxygen uptake at 37°C using a Gilson Differential Respirometer (Model G-14, Gilson Medical Electronics, Villiers-le-Bel, France) and was used to check some of the requirements for oxidase activity. The reagents were as described for Method A and were utilized according to Table II. After equilibration, the reaction was initiated by mixing

TABLE II

PROTOCOL FOR MANOMETRIC ASSAYS

Compartment	Volume per assay (ml) of reagent numbered in parenthesis according to text	
	D-Amino acid oxidase	L-Amino acid oxidase
Main vessel	1.2 ml buffer, pH 8.3 (1)	1.2 ml Buffer, pH 7.5 (1)
	0.1 ml D-alanine (7)	0.1 ml L-leucine (8)
	0.1 ml FAD (5)	0.1 ml water
Side-arm	0.1 ml sample	0.1 ml sample
Centre well	0.1 ml 2.5 M NaOH	0.1 ml 2.5 M NaOH

the contents of the main vessel and side-arm; the change in volume of the gas space was monitored for 30 min and recorded as μl per min.

Enzyme preparations

These were purchased from Sigma and comprised D-amino acid oxidase from hog kidney with activity approximately 0.02 unit per mg (Cat. No. A 9128), and L-amino acid oxidase from snake venom with activity approx. 0.65 unit per mg (Cat. No. A 4257).

Other amino acids

All were obtained from Sigma.

Calculation of Michaelis constants

These were determined by a weighted iterative least-squares method according to Wilkinson [21] employing a computer program [22].

Results

a) Stability and activity of glutamate dehydrogenase

Although certain aspects of this problem in relation to use of glutamate dehydrogenase as an enzyme indicator system have previously been investigated [23,24], it was desirable to examine others directly related to the amino acid oxidases. The stability of glutamate dehydrogenase in four buffers was studied by monitoring the increase in absorbance at 400 nm as precipitation of the enzyme occurred (Fig. 3); this wavelength was selected to eliminate the effect of spontaneous NADH oxidation. Instability was greatest around pH 8.0 and was least in glycylglycine and Tricine buffers. In all buffers, glutamate dehydrogenase was stabilised by addition of ADP or NaCl to final concentrations of 0.3 mM and 1.0 M, respectively, but such an effect was not shown by FMN or by FAD.

The effect of ADP, FMN and FAD on GDH activity in the same four buffers was studied (Table III). The well-recognised activation of GDH by ADP was demonstrated. In the absence of ADP, FMN was inhibitory, especially in glycylglycine and Tricine but FAD had no consistent effect. FAD, however

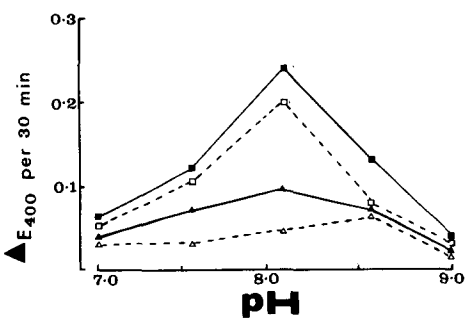

Fig. 3. Stability of glutamate dehydrogenase assessed by increase in A_{400nm} of solution containing 20 μl per 3 ml of the following buffers at a concentration of 0.1 M: ■, barbitone; □, Tris; ▲, Tricine; △, glycylglycine. Approx. 60% of activity was lost at the highest absorbance value shown.

TABLE III

EFFECT OF COENZYMES ON GLUTAMATE DEHYDROGENASE ACTIVITY

Data as $\Delta A_{340\,nm}$ per min at 37°C given by 10 µl glutamate dehydrogenase under conditions as outlined in Table I.

Buffer, pH 8.0	Coenzyme (final concentration of each 0.3 mM				
	None	ADP	FMN	FAD	ADP + FAD
Barbitone	0.10	0.44	0.14	0.20	0.37
Tris	0.10	0.42	0.06	0.06	0.35
Glycylglycine	0.26	0.66	0.08	0.24	0.53
Tricine	0.34	0.68	0.20	0.38	0.43

reduced glutamate dehydrogenase activity in the presence of ADP, moreso in glycylglycine and Tris, although activity was still greater than that obtained in the absence of ADP. From the standpoint of optimising glutamate dehydrogenase stability and activity, glycylglycine and Tricine seemed the most suitable buffers.

b) Optimal conditions for D-amino acid oxidase assay

Using Method B, activity was found to be maximal at pH 8.3 in glycylglycine buffer. This buffer provided slightly greater activity than Tricine, and 50% more than Tris or barbitone at that pH. The enzyme was "activated" by FAD and inhibited by ADP, but this inhibition could be overcome by increasing the concentration of FAD. Dialysis reduced activity and this could be restored by FAD. This is due to the comparatively weak binding of the FAD prosthetic group to the enzyme [8]. Activity reached a constant optimum between $6 \cdot 10^{-7}$ and $6 \cdot 10^{-5}$ M FAD. A concentration of $1 \cdot 10^{-5}$ M was chosen for the coupled assay, since this was 1/30 the concentration demonstrating inhibition of glutamate dehydrogenase in Table III.

The relationship between activity and the concentration of D-alanine was examined using Method A (Fig. 4). K_m was found to be 3.1 ± 0.12 mM. The final concentration of substrate definitively chosen for this method permits 85% of theoretical *V*.

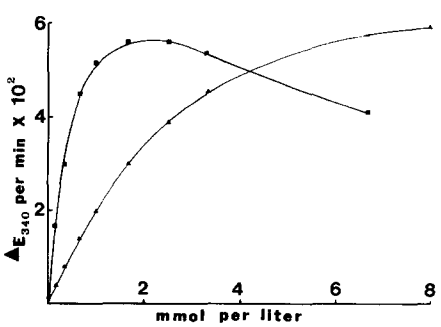

Fig. 4. Substrate—activity relationships of L-amino acid (■) and D-amino acid (▲) oxidases with L-leucine and D-alanine, respectively, utilising Method A.

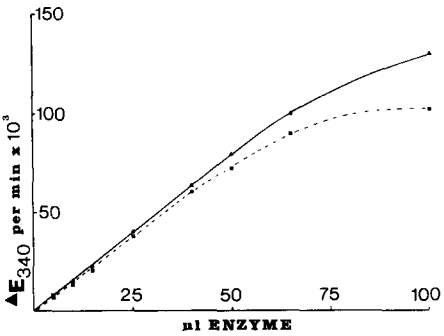

Fig. 5. Relationships of enzyme concentration to activity for L-amino acid (■) and D-amino acid (▲) oxidases utilising Method A.

c) Optimal conditions for L*-amino acid oxidase assay*

Using Method B, activity was maximal at pH 7.5. At this pH, activity was comparable in all four buffers (Tris, Tricine, barbitone, and glycylglycine) although glycylglycine was marginally superior. Neither FAD nor FMN increased activity even after dialysing the enzyme 48 h.

The relationship between activity and concentration of L-leucine was examined using Method A (Fig. 4). Although K_m was found to be 0.47 ± 0.09 mM, we chose a final concentration of 3.3 mM for the definitive assay in the light of the substrate inhibition demonstrated by L-leucine, and its limited solubility [25].

d) Characteristics of coupled assays

Linearity between reaction rate and enzyme concentration held for both assays to an absorbance change of at least 0.050 per min and fell away most rapidly with the L-amino acid oxidase assay (Fig. 5). Addition of the commercial enzyme preparations to human serum and 105 000 × *g* supernatants of human liver and kidney gave complete recovery, indicating lack of inhibitors or interfering side-reactions in these tissues.

Within-batch precision was assessed on samples with a range of activities and found to be very satisfactory (Table IV).

e) Substrate specificity

Each enzyme showed no reaction with amino acids of the opposite configuration, with one exception: D-amino acid oxidase showed with D-aspartate and L-aspartate 80% and 120%, respectively, of the activity in the presence of D-alanine. This was traced to contamination of each substrate by ammonia; an additional factor in the use of L-aspartate was generation of ammonia by the activity of other enzymes in the commercial preparation. When the substrate was passed through an ion-exchange column to remove ammonia, activity with D-aspartate fell to 30% of that with D-alanine. The necessity of using ammonia-free substrates cannot be emphasised too strongly. No activity with either form of aspartate could be demonstrated by Method B.

Table V presents data on the specificity of both enzymes using Method A. On the whole these results are comparable with those previously reported

TABLE IV

PRECISION OF COUPLED SPECTROPHOTOMETRIC ASSAYS AT THREE ACTIVITY LEVELS

Results based on ten replicate assays at each activity level under conditions of standard assay, data expressed as units per liter.

Enzyme	D-Amino acid oxidase	L-Amino acid oxidase
Mean	234	343
S.D.	0.8	4.1
C.V.*	0.3	1.2
Mean	69	93
S.D.	0.8	0.2
C.V.*	1.2	0.2
Mean	20	28
S.D.	1.3	1.0
C.V.*	6.5	3.6

* Coefficient of variation expressed as percentage.

[1,3,26] although, predictably, no action could be demonstrated with the amino acid proline which does not liberate ammonia on oxidation. Suggestions that the D-amino acid oxidases act upon glycine [26,27], D-tryphophan [26], and D-lysine [10], and that the L-amino acid oxidases act upon L-serine and L-threonine [10] as well as upon the L-isomers of alanine, valine, lysine and arginine [27] could not be confirmed with Method A. Results with Method B were in agreement with those of Method A except for the last statement above, slight activity being detected when L-amino acid oxidase acted upon alanine,

TABLE V

SPECIFICITY OF AMINO ACID OXIDASES AS DETERMINED IN COUPLED SPECTROPHOTOMETRIC ASSAY

Data for specific D- and L-isomers are given relative to D-alanine and L-leucine as 100 and in the same final concentrations, i.e. 17 mM for D-amino acids and 3.3 mM for L-amino acids. The isomers of glycine, lysine, proline, threonine and glutamic acid failed to yield measurable activity with their corresponding oxidase.

Amino acid	D-Amino acid oxidase	L-Amino acid oxidase
Alanine	100	0
Methionine	100	110
Aspartic acid	30	0
Tyrosine	80	65
Isoleucine	80	30
Phenylalanine	70	60
Leucine	60	100
Valine	50	0
Tryptophan	0	80
Cystine	0	40
Histidine	0	10
Arginine	1	1
Serine	1	0

valine, lysine and arginine. It is known that venoms of different species behave differently towards the various amino acid substrates and a particular example of this is seen with L-arginine. Lichtenberg and Wellner [27] and Guilbault and Hieserman [28] found only 30% and 12%, respectively, of the activity given by L-leucine with this substrate, whereas Massey and Curti [29] found it to be the most active substrate tested for L-amino acid oxidase from *Crotalus adamanteus*. Our results with the commercial preparation from this source are in accord with the earlier statement in the review by Meister and Wellner that, on the whole, L-arginine is not deaminated by the L-amino acid oxidases [26].

Discussion

The methods presented offer good precision, and linearity over a reasonably wide range. The need for standardisation does not arise because the calculation utilises the molar extinction coefficient of NADH. The contribution made by the "blank" will depend on the rate of endogenous ammonia formation; this will vary quite widely over a range of tissues and preparations.

The sensitivity of the new methods appears satisfactory, some comparative data being provided in Table VI. It would be possible to increase the sensitivity by a factor of 10^3 if fluorescence measurements, rather than absorption measurements were used to follow changes in the redox state of the pyridine nucleotides. Two options would be available: (a) kinetic monitoring of the decrease in NADH fluorescence at neutral pH; (b) measurement of the increased fluorescence due to newly formed NAD in strongly alkaline solution after terminating the enzyme reaction and destroying residual NADH [30]. End point fluorimetric methods have previously been described [27,31] and a

TABLE VI

COMPARISON OF SOME METHODS AVAILABLE FOR THE ASSAY OF AMINO ACID OXIDASES

Ref.	Specificity	Technique	Sensitivity (units/l) (lowest measurable activity)	Time of assay (min)	Substrate range*
1	D-, L-	Manometric	90	Variable	General
9	D-	Colorimetric	0.17	60	Restricted
10	D-, L-	Titrimetric	0.03	60	General
7	L-	Polarographic	0.6	Kinetic	General
31	D-	Fluorimetric	1.3	15	Restricted
27	D-, L-	Fluorimetric	0.3	15	General
28	D-, L-	Fluorimetric	0.05	Kinetic	General
15	L-	Spectrophotometric	0.5	Kinetic	Restricted
12	D-	Spectrophotometric	0.17	30	Restricted
16	D-	Spectrophotometric	138	Kinetic	Restricted
13	D-	Spectrophotometric	5	10	General
14	D-, L-	Spectrophotometric	30	15	Restricted
Present	D-, L-	Spectrophotometric	1.0	Kinetic	General

* By "general" it is implied that the method can be used with all amino acids as substrates; by "restricted" it is implied that the method can only be used with one or two amino acids or amino acid analogues.

kinetic fluorimetric assay [28] appears to be one of the most sensitive presented (Table VI).

Perhaps the principal advantage of the new methods lies in the ease with which they can be adapted for use with automatic enzyme rate analysers. This has been achieved for related ammonia-liberating enzymes of clinical interest [32—34]. Availability of such methods for the amino acid oxidases would be valuable in many experimental procedures requiring large numbers of assays as, for example, in monitoring column effluents during enzyme purification.

References

1 Blanchard, M., Green, D.E., Nocito, V. and Ratner, S. (1944) J. Biol. Chem. 155, 421—440
2 Bender, A.E. and Krebs, H.A. (1950) Biochem. J. 46, 210—219
3 Burton, K. (1955) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. 2, pp. 199—204, Academic Press, New York
4 Nagatsu, T. and Yagi, K. (1966) J. Biochem. Tokyo 60, 219—221
5 Dixon, M. and Kleppe, K. (1965) Biochim. Biophys. Acta 96, 357—367
6 Yagi, K., Naoi, M., Harada, M., Okamura, K., Hidaka, H., Ozawa, T. and Kotaki, A. (1967) J. Biochem. Tokyo 61, 580—597
7 Luppa, D. and Aurich, H. (1971) Enzyme 12, 688—693
8 Meister, A., Wellner, D. and Scott, S.J. (1960) J. Natl. Cancer Inst. 24, 31—49
9 Corrigan, J.J., Wellner, D. and Meister, A. (1963) Biochim. Biophys. Acta 73, 50—56
10 Scannone, H., Wellner, D. and Novogrodsky, A. (1964) Biochemistry 3, 1742—1745
11 Wellner, D. and Meister, A. (1960) J. Biol. Chem. 235, 2013—2018
12 Dunn, J.T. and Perkoff, G.T. (1963) Biochim. Biophys. Acta 73, 327—331
13 Soda, K. (1968) Anal. Biochem. 25, 228—235
14 Wellner, D. and Lichtenberg, L.A. (1971) in Methods in Enzymology (Tabor, H. and Tabor, C.W., eds), Vol. 17B, pp. 593—596, Academic Press, New York
15 Weissbach, H., Robertson, A.V., Witkop, B. and Udenfriend, S. (1960) Anal. Biochem. 1, 286—290
16 Fonda, M.L. and Anderson, B. (1967) J. Biol. Chem. 242, 3957—3962
17 Ellis, G., Belfield, A. and Goldberg, D.M. (1970) in 7th International Congress of Clinical Chemistry (Frei, J. and Jemelin, M., eds), Vol. 2, pp. 95—107, Karger, Basel
18 Ellis, G. and Goldberg, D.M. (1970) J. Lab. Clin. Med. 76, 507—517
19 Ellis, G. and Goldberg, D.M. (1972) Anal. Lett. 5, 65—73
20 Ellis, G. and Goldberg, D.M. (1972) Biochem. Med. 6, 380—391
21 Wilkinson, G.N. (1961) Biochem. J. 80, 324—332
22 Hoy, T.G. and Goldberg, D.M. (1971) Int. J. Biomed. Comput. 2, 71—77
23 Di Prisco, G. and Strecker, H.J. (1966) Biochim. Biophys. Acta 122, 413—422
24 Ellis, G. and Goldberg, D.M. (1972) Clin. Chim. Acta 39, 472—474
25 In Handbook of Chemistry and Physics (Weast, R.C., ed.), 48th edn, p. C705, Chemical Rubber Company, Cleveland, Ohio, 1967
26 Meister, A. and Wellner, D. (1963) in The Enzymes (Boyer, P.D., Lardy, H. and Myrback, K., eds), 2nd edn, Vol. 7, pp. 609—648, Academic Press, New York
27 Lichtenberg, L.A. and Wellner, D. (1968) Anal. Biochem. 26, 313—319
28 Guilbault, G.G. and Hieserman, J.E. (1968) Anal. Biochem. 26, 1—11
29 Massey, V. and Curti, B. (1967) J. Biol. Chem. 242, 1259—1264
30 Lowry, O.H. and Passonneau, J.V. (1972) in A Flexible System of Enzymatic Analysis, pp. 3—20, Academic Press, New York
31 Verity, M.A., Gallagher, R. and Brown, W.J. (1967) Biochem. J. 103, 375—381
32 Goldberg, D.M., Ellis, G. and Wilcock, A.R. (1971) Ann. Clin. Biochem. 8, 189—194
33 Goldberg, D.M. and Ellis, G. (1972) J. Clin. Pathol. 25, 907—909
34 Ellis, G., Spooner, R.J. and Goldberg, D.M. (1973) Clin. Chim. Acta 47, 78—87