Fluorimetric Determination of D-Amino Acid Oxidase

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1. A sensitive fluorimetric procedure for the assay of D-amino acid oxidase has been developed. 2. The method depends on the formation of a fluorescent derivative, 2-hydroxy-3-methylquinoxaline, on condensation of pyruvate with *o*-phenylenediamine in acid medium. 3. 2-Hydroxy-3-methylquinoxaline fluoresces strongly in 50% (v/v) sulphuric acid after excitation at 375m μ . A single emission peak is observed at 480m μ . 4. Formation of the quinoxaline is dependent on time, temperature, acidity and relative concentration of reactants. 5. A particulate preparation from mouse kidney required FAD for optimum activity at pH 8.5; K_m was 3.8×10^{-3} M; K_{FAD} was 1.4×10^{-7} M and the reaction was strongly inhibited by *p*-chloromercuribenzoate and phenylmercuric acetate. 6. Subcellular fractionation on a sucrose density gradient confirmed that the D-amino acid oxidase was localized on small granules.

The oxidative deamination of D-amino acids to the corresponding α -oxo acid and ammonia or amine is catalysed by D-amino acid oxidases (EC 1.4.3.3; D-amino acid-oxygen oxidoreductase). Previous assays of enzymic activity have depended on the rate of oxygen consumption, measured manometrically in a Warburg apparatus (Krebs, 1933; Hellerman, Coffey & Neims, 1965) or the spectrophotometric measurement of an α -oxo acid, in particular pyruvate (Friedemann & Haugen, 1943; Tonhazy, White & Umbreit, 1950). These methods have proved tedious for routine analyses and have utilized incubation periods of 2–3 hr. or longer for tissue particulate fractions (Baudhuin *et al.* 1964). Further, the enzyme reaction has not proved linear over such long incubation periods.

Recent observations have indicated the close association of uricase, D-amino acid oxidase and catalase with subcellular particles. During investigations on the particulate localization of mouse-liver uricase, presumably associated with the microbody (Wattiaux, Wibo & Baudhuin, 1963; Beaufay *et al.* 1964; Verity, Reith, Ho & Brown, 1965) a rapid sensitive procedure for the assay of particulate D-amino acid oxidase was needed. The method reported below fulfils these criteria and depends on the formation of fluorescent quinoxalines on condensation of α -oxo acids with *o*-phenylenediamine in acid solution. Such a derivative of pyruvic acid, 2-hydroxy-3-methylquinoxaline, is highly fluorescent and stable in concentrated acid (Spikner & Towne, 1962).

MATERIALS AND METHODS

Solutions of *o*-phenylenediamine dihydrochloride in 3N-H₂SO₄ (Eastman Chemical Co., Rochester, N.Y., U.S.A.) were prepared freshly each day. Preliminary experiments demonstrated the unsuitability of using the dihydrochloride of chloro-*p*-phenylenediamine for condensation. Chromatographically homogeneous D-alanine, [α]_D –14.5°, was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Sodium pyruvate (K. & K. Laboratories Inc., Plainview, N.Y., U.S.A.) was used without further purification. FAD (monosodium salt) was obtained from California Corp. for Biochemical Research and prepared each week as a 0.1M-stock solution and was kept in the dark. This FAD contained no detectable FMN, but contains 9% of riboflavin as a contaminant, an amount insufficient to influence the kinetics (Massey & Swoboda, 1963). Crystalline D-amino acid oxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) obtained from swine kidney has an activity of approx. 0.025 mole of O₂ taken up/min./mg. of crude enzyme at 37°.

Horse-heart cytochrome *c* supplied as an amorphous powder was obtained from Mann Research Laboratories, New York, N.Y., U.S.A., and dissolved in 0.03M-phosphate buffer before use. Phenylmercuric acetate and *p*-chloromercuribenzoate were dissolved in a small amount of 0.05N-NaOH and diluted with 0.05M-pyrophosphate buffer, pH 8.3. All other chemicals were reagent grade when available.

Preparation of mouse-kidney apoenzyme. After decapitation, both kidneys were removed rapidly, placed in ice-cold 0.25M-sucrose and homogenized in a smooth-walled glass Potter-Elvehjem homogenizer for 30 sec. at 350 rev./min. The homogenate was centrifuged at 750g for 10 min. in a Spinco model L ultracentrifuge. The supernatant was spun

at 17000g for 10 min. and the resulting pellet resuspended in 0.25M-sucrose and re-spun again under the same conditions. The 'large-granule' pellet was suspended finally in 0.05M-pyrophosphate buffer, pH 8.3, to give a final protein concentration of 1-2 mg./ml.

Subcellular fractions were prepared by differential and density-gradient centrifugation. Two kidneys were homogenized in 10 ml. of ice-cold 0.25M-sucrose containing EDTA (1 mM). The nuclear fraction was obtained at 6×10^3 g-min. in the Spinco rotor no. 50, washed and the washing added to the supernatant. Forces are expressed as time integrals of the maximum field where $R_{\max}$ is 7.1 cm. (de Duve & Berthet, 1953). The 'mitochondrial' fraction was spun at 8.5×10^4 g-min. The pellet was re-suspended, spun at 8.5×10^4 g-min. and the washing added to the 'mitochondrial' fraction supernatant, which was centrifuged ultimately at 3.35×10^6 g-min., providing microsomal and cell-sap fractions. All fractions were suspended in 0.05M-pyrophosphate buffer before assay. A partial separation of mitochondria, lysosomes and microbodies was made on a three-step discontinuous sucrose density gradient (0.3M-0.6M-1.2M). The 'mitochondrial' fraction from differential centrifugation was resuspended in 1.5 ml. of 0.3M-sucrose and layered on cold 0.6M-sucrose as the intermediate layer. The gradient was centrifuged at 1.07×10^5 g-min. ($R_{\max}$ 9.8 cm.) in a Spinco swinging bucket, rotor no. 39, providing three easily discernible layers, A, B and C, and pellet D.

Reference enzymes. Activation of lysosomal and mitochondrial enzymes occurred in the presence of 0.1% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.). Mitochondrial cytochrome *c* oxidase was assayed by a slight modification of the spectrophotometric method of Cooperstein & Lazarow (1951), in which the 0.03M-phosphate buffer was made iso-osmotic by addition of 0.15M-KCl. Enzyme activity is expressed as $\Delta E/\text{min.}/\text{mg.}$ of protein. Acid-phosphatase and β -glucuronidase activities were measured by the fluorimetric procedures of Verity & Brown (1964) and Verity, Caper & Brown (1964). Protein was determined by the method of Lowry & Lopez (1951).

Fluorimetric assay of D-amino acid oxidase. The procedure finally developed is described. The reaction mixture contained 0.5 ml. of 0.05M-pyrophosphate buffer, pH 8.3, 0.2 ml. of 0.2M-D-alanine and 50 μ l. of 32 μ M-FAD. The reaction was started in duplicate by addition of 0.1 ml. of enzyme solution and stopped by the addition of 0.1 ml. of 0.8N-trichloroacetic acid after incubation at 37° for 15 min. A zero-time internal blank was obtained by immediate acidification of one tube containing reaction mixture after the addition of enzyme. After centrifugation and precipitation of protein, 0.5 ml. of the clear supernatant was added to a 5 ml. Pyrex volumetric tube containing 1.0 ml. of mM-*o*-phenylenediamine dihydrochloride freshly prepared in 3N-H₂SO₄. Pyruvate standards and an *o*-phenylenediamine blank were prepared by adding 0.5 ml. of 0.05M-pyrophosphate buffer containing a known pyruvate concentration or no pyruvate to 1.0 ml. of mM-*o*-phenylenediamine. Formation of the fluorescent 2-hydroxy-3-methylquinoxaline proceeded in a 60° water bath for 1 hr. The Pyrex tubes were lightly capped with glass stoppers to prevent excessive evaporation. The tubes were cooled. Then 1.0 ml. of 50% (v/v) H₂SO₄ was added, and mixed for 3-5 min. at room temperature before transference to a fused quartz cuvette (10 mm. $\times$ 10 mm. $\times$

48 mm.). Fluorescence was measured in a Farrand spectrofluorimeter equipped with a 150 w xenon light-source and RCA ultrasensitive microammeter by using an excitation wavelength of 375 m μ and emission wavelength of 480 m μ .

RESULTS

Condensation reaction of pyruvate with o-phenylenediamine. The overall reaction sequence of the oxidative deamination of D-alanine suggests the uptake of 1 mol. of oxygen/mol. of pyruvate formed:

Hence the formation of pyruvate is stoichiometrically related to the deamination of D-alanine, even in the absence of catalase.

The excitation and emission fluorescence spectra of the end product, 2-hydroxy-3-methylquinoxaline resulting from *o*-phenylenediamine condensation with pyruvate, are plotted in Fig. 1. Equal volumes of 1 mM-*o*-phenylenediamine and sodium pyruvate solution (0.1 μ mole/ml., in 3N-sulphuric acid) were heated in a 60° water bath for 60 min. The fluorescence spectra were obtained in the presence of 50% (v/v) sulphuric acid. A single primary excitation peak is noted at 375 m μ (recording emission wavelength, 470 m μ), and a corresponding single emission peak observed at 480 m μ (excitation wavelength, 375 m μ). The fluorescent end product was stable in 50% (v/v) sulphuric acid over a period of 3 hr. at room temperature. The rate of formation of the quinoxaline derivative was dependent on the degree of acidity, temperature and time of condensation. Fig. 2

Fig. 1. Corrected excitation (●) and emission (○) spectra for 2-hydroxy-3-methylquinoxaline in the presence of 50% (v/v) H₂SO₄. The condensation reaction mixture (2 ml.) contains 0.5 mM-*o*-phenylenediamine and 0.1 μ mole of sodium pyruvate in 3N-H₂SO₄, heated at 60° for 60 min.

Fig. 2. Condensation reaction rates for 0.03 μ mole (○) and 0.09 μ mole (●) of sodium pyruvate with 0.5 mM *o*-phenylenediamine in 3N- H_2SO_4 . Fluorescence (μA) was measured in 50% (v/v) H_2SO_4 at excitation wavelength 375 m μ and emission wavelength 480 m μ .

Concn. of pyruvate (μ mole/2.0 ml. of condensation system)

Fig. 3. Variation in fluorescence (μA) with increasing pyruvate concentration in the condensation reaction with different concentrations (Δ , 0.13 mM; ○, 0.25 mM; ●, 0.5 mM) of *o*-phenylenediamine in 3N- H_2SO_4 . Fluorescence parameters are as given in Fig. 2.

suggests that the reaction is not fully complete even after 2½ hr. in 3N-sulphuric acid, but indicates that approx. 90% of the asymptote fluorescence is obtained after 60 min. A linear fluorescence relationship is noted as a function of pyruvate concentration when less than 0.1 μ mole of pyruvate is present with 0.5 mM *o*-phenylenediamine (Fig. 3). At *o*-phenylenediamine concentrations less than

Fig. 4. Effect of FAD (0.4 μM) on the proportional relationship between fluorescence and concentration of crystalline enzyme protein in the assay system (see the Materials and Methods section). ●, 0.4 μM -FAD; ○, no FAD.

0.5 mM the fluorescence intensity decreases more markedly with increasing pyruvate concentrations. No condensation occurs in the presence of 0.1 M-phosphate buffer, pH 8.3 (Spikner & Towne, 1962).

Effect of time of incubation, pH and concentration of enzyme on the assay of D-amino acid-oxidase activity. Studies on the enzymic conversion of D-alanine into pyruvate by commercial crystalline preparations of D-amino acid oxidase (see the Materials and Methods section for assay details) demonstrated a strict linearity between the time of incubation and fluorescence of the liberated pyruvate in the supernatant after precipitation by trichloroacetic acid. The fluorescence, which is strictly proportional to the amount of pyruvate, revealed a non-linear proportionality to enzyme concentration (Fig. 4). Strict linearity at all concentrations was found on addition of FAD (0.4 μM), indicating that a small amount of FAD was bound to commercial D-amino acid apoenzyme, with correspondingly low initial reaction rates occurring at very low enzyme concentrations (Fig. 4).

Previous manometric investigations of partially purified D-amino acid oxidase have revealed a pH optimum of 8.5–8.8 (Krebs, 1935; Negelein & Bromel, 1939; Frisell & Hellerman, 1957). Comparative pH-activity curves (Fig. 5) in 0.05 M-sodium pyrophosphate-hydrochloric acid buffer were obtained for crystalline D-amino acid oxidase and an enzyme preparation derived from the 17000g pellet in the presence of 1 μM -FAD. Broad pH optima were obtained with both preparations, with an optimum at $\text{pH } 8.5 \pm 0.2$. The tissue preparation showed a slight shift to a more acid range.

Recovery of pyruvate from the assay system. There was almost quantitative recovery of added pyruvate in the supernatant from the trichloroacetic acid precipitation of large-granule-fraction protein

(Table 1). The standard deviation of the values at the two concentrations of pyruvate (0.04 and 0.1 μ mole) were different, but the coefficient of variance obtained from the standard error of the means were comparable. The significant fluorescence value of the tissue blank is attributed to a small amount of endogenous pyruvate in the large-granule fraction.

Kinetic and inhibitor studies of D-amino acid-oxidase activity. Kinetic data were obtained from determinations of initial enzyme velocity at increasing substrate concentrations. Near-maximum velocity ($V_{\max}$) was obtained at 50mM-D-alanine. No substrate inhibition was apparent at 0.15M. K_m values for crystalline D-amino acid

oxidase and 'large-granule' enzyme in the presence of 2 μ M-FAD and pyrophosphate buffer, pH 8.3, gave values of 9.1×10^{-3} M and 3.8×10^{-3} M respectively, calculated from Lineweaver-Burk plots (Fig. 6). Though it is recognized that the K_m value will depend on the degree of enzyme purity, nature of substrate, pH and type of buffer used, the above values are comparable with those of 6.1×10^{-3} – 7.1×10^{-3} M obtained by Hellerman, Lindsay & Bovarnick (1946), Klein (1953) and Gabay & Harris (1965) with purified enzyme preparations.

The enzymic activity of D-amino acid oxidase as a function of FAD concentration is shown in a double-reciprocal plot of $1/v$ versus $1/[FAD]$ (Fig. 7). The determined K_{FAD} value of 1.4×10^{-7} M agrees well with the published values of 1.82×10^{-7} M (Gabay & Harris, 1965) and 1.3×10^{-7} M (Burton, 1951) obtained from manometric studies.

Fig. 5. pH-activity profiles for the fluorimetric determination of D-amino acid-oxidase activity of crystalline enzyme obtained from swine kidney (○) and a preparation obtained from the 17000g fraction from mouse kidney (●). The reaction mixture (0.85ml.) contained a pyrophosphate buffer (0.05M), D-alanine (47mM), FAD (1 μ M) and enzyme preparation (0.1ml.), and was incubated at 37° for 15 min. The condensation reaction and fluorescence measurements were carried out as described in the Materials and Methods section.

Fig. 6. Double-reciprocal plots of initial enzyme velocity and D-alanine concentration for crystalline D-amino acid oxidase (●) and 17000g fraction from mouse kidney (×). The assay system contained pyrophosphate buffer, pH 8.3 (0.05M), and FAD (2 μ M).

Table 1. Recovery of pyruvate in trichloroacetic acid extracts of mitochondrial fractions

Pyruvate was added to 1 ml. of mitochondrial fractions suspended in 0.05M-pyrophosphate buffer. Samples (0.5ml.) of supernatant were obtained after precipitation with cold trichloroacetic acid and condensed with 1.0ml. of o-phenylenediamine. Fluorescence was measured in arbitrary meter units. The small amount of fluorescence present in the supernatant after mitochondrial precipitation probably represents endogenous mitochondrial pyruvate. Reproducibility is given as mean $\pm$ s.e.m., with the coefficient of variance in parenthesis. The coefficient of variance is calculated from the standard deviation/mean.

Pyruvate added (μ mole)	Fluorescence after condensation		Reproducibility	Recovery (%)
	No tissue	Tissue		
0	—	0.048	—	—
0.04	0.31	0.31, 0.35, 0.32	0.33 ± 0.01 (0.67)	91
0.1	0.76	0.86, 0.74, 0.80	0.80 ± 0.03 (0.74)	98

Addition of *p*-chloromercuribenzoate or phenylmercuric acetate to a washed 'large-granule' preparation of kidney D-amino acid oxidase demonstrated the probable thiol nature of the enzyme (Table 2). Moreover, the protective role of FAD when present at the same time as the inhibitor (Table 2) was demonstrated. Simultaneous preincubation in the presence of inhibitor and 0.8 mM-L-cysteine considerably antagonized the irreversible inhibition of the thiol inhibitors. Of note is the greater protection conferred against phenylmercuric acetate as compared with *p*-chloromercuribenzoate, providing some evidence for the dual inhibitory nature of this compound as a thiol-reactive agent and as a substituted benzoate manifesting substrate competitive inhibition (Bartlett, 1948; Frisell & Hellerman, 1957).

Intracellular distribution of D-amino acid oxidase in mouse kidney. A localization in mitochondria has been reported for D-amino acid oxidase in rat

liver (Dianzani, 1954). However, Paigen (1954) demonstrated a predominant localization of the enzyme in the light-mitochondrial fraction, in contrast with the succinoxidase system, which is more abundant in the heavier fraction. The localization of D-amino acid oxidase, assayed by the fluorimetric procedure, in the 'mitochondrial' fraction of kidney homogenates was confirmed in these experiments (Table 3). Assays were performed in the presence of 2 μ M-FAD, the pellets being resuspended in buffer (0.05 M-pyrophosphate containing 0.1% of Triton X-100), because of interference with the *o*-phenylenediamine condensation reaction in the presence of 0.25 M-sucrose. Further characterization of the particulate localization of D-amino acid oxidase was provided by the density-gradient fractionation of the mitochondrial fraction (Table 4). In this study, a distinct separation

Fig. 7. Double-reciprocal plot of D-amino acid-oxidase activity, obtained from 17000g fraction of mouse kidney as a function of FAD concentration. The assay system was as described in the Materials and Methods section with FAD (50 μ l.) added to give the indicated final concentration.

Table 2. Inhibition of washed kidney 'mitochondrial' D-amino acid oxidase by *p*-chloromercuribenzoate and phenylmercuric acetate

Samples of 'mitochondrial' suspensions treated with Triton X-100 were placed in 0.05 M-pyrophosphate buffer to which inhibitor, L-cysteine or FAD (5 μ M) was added to give a final volume of 0.6 ml. After 5 min. at 24° the enzyme reaction was initiated by addition of D-alanine (0.06 M). FAD (5 μ M) was added to the control system of the apoenzyme-FAD experiment after the 5 min. preincubation. The values given represent final concentrations in the assay medium. Percentage inhibition is the mean of two experiments in which parallel reactions are run without inhibitor.

Inhibitor	Inhibition (%)	
	Apoenzyme	Apoenzyme + FAD
<i>p</i> -Chloromercuribenzoate (55 μ M)	88	65
Phenylmercuric acetate (70 μ M)	91	48
<i>p</i> -Chloromercuribenzoate (55 μ M) + L-cysteine (0.8 mM)	26	30
Phenylmercuric acetate (70 μ M) + L-cysteine (0.8 mM)	8	3

Table 3. Localization of D-amino acid oxidase in subcellular fractions of mouse kidneys

Experimental details are given in the Materials and Methods section. Values represent the mean activities $\pm$ S.E.M. of four experiments.

Fraction	Protein (mg./g. wet wt. of homogenate)	D-Amino acid-oxidase activity (μ moles of pyruvate/min./fraction)
Homogenate	151.7	40.8 $\pm$ 3.2
Nuclear	42.7	6.41 $\pm$ 0.34
Mitochondrial	29.9	24.8 $\pm$ 2.3
Microsomal	23.5	3.06 $\pm$ 0.46
Supernatant	48.8	4.88 $\pm$ 0.30

Table 4. *Specific activity of particulate D-amino acid oxidase after density-gradient subfractionation of the 'mitochondrial' fraction obtained from mouse kidney*

The 'mitochondrial' pellet obtained at $8.5 \times 10^4 g$ -min. was resuspended in 0.3 M-sucrose and layered over a discontinuous 0.6–1.2 M-sucrose gradient (see the Materials and Methods section). Separated layers were spun at 17000g for 10 min., and pellets resuspended in 0.05 M-pyrophosphate buffer containing 0.1% (v/v) of Triton X-100. Values are means $\pm$ s.e.m. of four determinations.

Density-gradient fraction	D-Amino acid oxidase (μ moles of pyruvate/ mg. of protein/min.)	Acid phosphatase (μ moles/100 mg. of protein/min.)	β -Glucuronidase (μ moles/100 mg. of protein/hr.)	Cytochrome <i>c</i> oxidase (ΔE /mg. of protein/min.)
A	0.97 ± 0.08	2.17 ± 0.33	7.12 ± 0.51	0.65 ± 0.04
B	2.83 ± 0.41	5.53 ± 0.38	13.16 ± 0.80	0.80 ± 0.04
C	1.70 ± 0.11	2.62 ± 0.26	7.80 ± 0.47	3.3 ± 0.31
D	0.26 ± 0.03	1.49 ± 0.18	3.50 ± 0.30	2.63 ± 0.47

between mitochondrial cytochrome *c* oxidase, and the lysosomal enzymes, acid phosphatase and β -glucuronidase, was apparent. However, the gradient reveals the close association of D-amino acid oxidase with the lysosomal-'microbody' fraction but does not provide for a more definitive localization in the 'microbody'.

DISCUSSION

The general reaction for the formation of quinoxaline derivatives from the condensation of α -oxo acids with *o*-phenylenediamine was originally described by Hinsberg (1887) and subsequently shown to be an almost quantitative reaction by Billman & Rendall (1944), who obtained over 90% yields. In the present experiments, the condensation product with pyruvate in an acid medium results in the formation of 2-hydroxy-3-methyl-quinoxaline:

Condensation in 2N-sulphuric acid was adequate and preferred in that the quinoxaline product was stabilized against oxidation. Fluorescence was determined at the optimum wavelength of excitation and emission in the presence of 50% (v/v) sulphuric acid as the high acidity increased the intensity of fluorescence. The small amounts of pyruvate measured in the reaction are advantageous as maximal fluorescence yields are only obtained in dilute solutions of α -oxo acid and *o*-phenylenediamine, owing to the formation of interfering

high-molecular-weight polymeric substances when concentrated α -oxo acid solutions are used (Billman & Rendall, 1944). A linear fluorescence plot (Fig. 3) of pyruvate concentration (0.01–0.14 μ mole) at constant *o*-phenylenediamine concentration suggests that polymer formation or *o*-phenylenediamine exhaustion are non-contributory factors.

Among the advantages of a fluorimetric method are the decreased sample size with the increased sensitivity of the procedure, the use of low substrate concentrations allowing a closer approximation to first-order kinetics, the decrease in reaction period with increase in speed of analysis and maintained linearity over the period of incubation. The K_m value ($9.1 \times 10^{-3} M$) obtained with the crystalline enzyme agrees favourably with the values obtained from manometric studies. Similarly, the K_{FAD} value of $1.4 \times 10^{-7} M$ is comparable with a published value of $1.3 \times 10^{-7} M$ (Burton, 1951).

The enzymic activity in a partially purified preparation obtained from a washed large-granule fraction of mouse kidney is depressed by a number of thiol-reacting compounds. The procedure and particulate enzyme are thus capable of confirming previous studies of the dependence of the apoenzyme on thiol groups (Frisell & Hellerman, 1957). Moreover, the partial protection offered by FAD during simultaneous preincubation with mercurials (Frisell & Hellerman, 1957; Yagi & Ozawa, 1961; Hellerman *et al.* 1965) suggests that FAD is bound to the enzyme by thiol groups. This view has, however, been questioned (Frisell & Hellerman, 1957).

Combined biochemical and morphological studies of liver fractions (Baudhuin *et al.* 1964; Baudhuin, Beaufay & de Duve, 1965; Verity *et al.* 1965) have revealed a population of particles enriched in catalase, urate oxidase and D-amino acid oxidase. The latter two enzymes within these particles, termed 'microbodies', 'uricosomes' or 'peroxisomes', do not show enzyme latency, in contrast with all the lysosomal hydrolases. Catalase is an

exception in this respect (Adams & Burgess, 1957; Baudhuin *et al.* 1964). Investigations of latency properties of these particle-linked enzymes is dependent on particle purification, rapidity and sensitivity of assay under standardized iso-osmotic conditions (de Duve, 1963). The fluorimetric procedure for D-amino acid oxidase presented above fulfils many of these criteria.

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REFERENCES

- Adams, D. H. & Burgess, E. A. (1957). *Brit. J. Cancer*, **11**, 310.
- Bartlett, G. R. (1948). *J. Amer. chem. Soc.* **70**, 1010.
- Baudhuin, P., Beaufay, H. & de Duve, C. (1965). *J. Cell Biol.* **28**, 219.
- Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P. & de Duve, C. (1964). *Biochem. J.* **92**, 179.
- Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J. & de Duve, C. (1964). *Biochem. J.* **92**, 184.
- Billman, J. H. & Rendall, J. L. (1944). *J. Amer. chem. Soc.* **66**, 540.
- Burton, K. (1951). *Biochem. J.* **48**, 458.
- Cooperstein, S. J. & Lazarow, A. (1951). *J. biol. Chem.* **189**, 665.
- de Duve, C. (1963). *Ciba Found. Symp.: Lysosomes*, p. 1. Ed. by de Reuck, A. V. S. & Cameron, M. P. Boston: Little, Brown and Co.
- de Duve, C. & Berthet, J. (1953). *Nature, Lond.*, **172**, 1142.
- Dianzani, M. V. (1954). *G. Biochim.* **3**, 29.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Frisell, W. R. & Hellerman, L. (1957). *J. biol. Chem.* **225**, 53.
- Gabay, S. & Harris, S. R. (1965). *Biochem. Pharmacol.* **14**, 17.
- Hellerman, L., Coffey, D. S. & Neims, A. H. (1965). *J. biol. Chem.* **240**, 290.
- Hellerman, L., Lindsay, A. & Bovarnick, M. R. (1946). *J. biol. Chem.* **163**, 553.
- Hinsberg, O. (1887). *Liebigs Ann.* **237**, 327.
- Klein, J. R. (1953). *J. biol. Chem.* **205**, 725.
- Krebs, H. A. (1933). *Hoppe-Seyl. Z.* **217**, 191.
- Krebs, H. A. (1935). *Biochem. J.* **29**, 1620.
- Lowry, O. H. & Lopez, J. A. (1951). *J. biol. Chem.* **193**, 265.
- Massey, V. & Swoboda, B. E. P. (1963). *Biochem. Z.* **338**, 474.
- Negelein, E. & Bromel, H. (1938). *Biochem. Z.* **300**, 225.
- Paigen, K. (1954). *J. biol. Chem.* **206**, 945.
- Spikner, J. E. & Towne, J. C. (1962). *Analyt. Chem.* **34**, 1468.
- Tonhazy, N. E., White, N. G. & Umbreit, W. W. (1950). *Arch. Biochem.* **28**, 36.
- Verity, M. A. & Brown, W. J. (1964). *Exp. Cell Res.* **35**, 84.
- Verity, M. A., Caper, R. & Brown, W. J. (1964). *Arch. Biochem. Biophys.* **106**, 386.
- Verity, M. A., Reith, A., Ho, Y. K. & Brown, W. J. (1965). *Fed. Proc.* **24**, 680.
- Wattiaux, R., Wibo, M. & Baudhuin, P. (1963). *Ciba Found. Symp.: Lysosomes*, p. 176. Ed. by de Reuck, A. V. S. & Cameron, M. P. Boston: Little, Brown and Co.
- Yagi, K. & Ozawa, T. (1961). *Biochim. biophys. Acta* **42**, 381.