Purification and Properties of Urease from Bovine Rumen

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Urease (urea amidohydrolase, EC 3.5.1.5) was extracted from the mixed rumen bacterial fraction of bovine rumen contents and purified 60-fold by $(\text{NH}_4)_2\text{SO}_4$ precipitation, calcium phosphate-gel adsorption and chromatography on hydroxyapatite. The purified enzyme had maximum activity at pH 8.0. The molecular weight was estimated to be 120000–130000. The K_m for urea was $8.3 \times 10^{-4} \text{ M} \pm 1.7 \times 10^{-4} \text{ M}$. The maximum velocity was $3.2 \pm 0.25 \text{ mmol of urea hydrolysed/h per mg of protein}$. The enzyme was stabilized by 50 mM-dithiothreitol. The enzyme was not inhibited by high concentrations of EDTA or phosphate but was inhibited by Mn^{2+} , Mg^{2+} , Ba^{2+} , Hg^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} , Ni^{2+} and Co^{2+} . *p*-Chloromercuribenzenesulphonate and *N*-ethylmaleimide inhibited the enzyme almost completely at 0.1 mM. Hydroxyurea and acetohydroxamate reversibly inhibited the enzyme. Polyacrylamide-gel electrophoresis showed that the mixed rumen bacteria produce ureases which have identical molecular weights and electrophoretic mobility. No multiple forms of urease were detected.

The extensive literature on jack-bean urease (urea amidohydrolase, EC 3.5.1.5) has been reviewed and summarized by Reithel (1971). Apart from plant sources, ureases occur in a number of micro-organisms (Seneca *et al.*, 1962). In contrast with the plant enzyme, only a few microbial ureases have been purified, mainly because of their unstable nature. These include ureases from *Proteus vulgaris* (Larson & Kallio, 1954) and *Proteus mirabilis* (Andersen *et al.*, 1969).

Urease activity in the rumen fluid was studied by Pearson & Smith (1943). Unlike ureases from plant and other microbial sources, for which no physiological function is known, the urease activity in the rumen serves an important physiological function. This urease is responsible for hydrolysis of urea that is recycled through the saliva or present in the diet (Allison, 1970). In spite of this importance, very little is known about the properties of urease isolated from rumen bacteria (rumen urease). Pearson & Smith (1943) reported that attempts to extract active urease from rumen organisms in a soluble form were unsuccessful. Jones *et al.* (1964) reported on the properties of urease extracted from acetone-dried preparations of rumen bacteria. The activity in these extracts was very low. Partial purification of rumen urease has also been reported by Baintner (1964) and by Brent *et al.* (1971). Mahadevan *et al.* (1976) have described the properties of urease present in crude extracts of rumen bacteria. The present paper deals with the purification of rumen urease and some of the properties of the purified enzyme.

Experimental

Materials

The sources of most of the materials used have been described (Mahadevan *et al.*, 1976). Thyroglobulin and γ -globulin were from Mann Research Laboratories, New York, NY, U.S.A. Bovine serum albumin was from Nutritional Biochemicals, Cleveland, OH, U.S.A. Sperm-whale myoglobin was from Miles Laboratories, Kankakee, IL, U.S.A. Horse heart cytochrome *c* and rabbit muscle enolase were from Boehringer, Montreal, Que., Canada. Ox liver catalase was from Calbiochem, La Jolla, CA, U.S.A. Rabbit muscle aldolase and Sephadex G-200 were from Pharmacia, Dorval, Que., Canada. The apparatus and chemicals used in polyacrylamide-gel electrophoresis were from Canalco Ltd., Ottawa, Ont., Canada. Calcium phosphate gel and hydroxyapatite were from Bio-Rad, Mississauga, Ont., Canada.

Methods

Collection of rumen fluid, isolation of bacterial fraction, solubilization and partial purification of urease. The procedures used in these steps have been described in detail previously (Mahadevan *et al.*, 1976). The enzyme eluted from the calcium phosphate gel was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation. The precipitate was collected by centrifugation at 17000*g* for 30 min and dissolved in a small volume of 10 mM-potassium phosphate buffer, pH 6.8, containing 10 mM-dithiothreitol.

Hydroxyapatite - column chromatography. A hydroxyapatite column (1.5cm×6.0cm) was equilibrated with 50ml of 10mM-potassium phosphate buffer, pH6.8, containing 10mM-dithiothreitol. The enzyme obtained from the calcium phosphate-gel-adsorption step was applied to the column. The column was eluted with 30ml of the starting buffer at a flow rate of 9.0ml/h and fractions (5ml) were collected. Most of the protein was eluted with the starting buffer. A further washing with 15 ml of 20mM-phosphate buffer, pH6.8, containing 10mM-dithiothreitol removed more inactive protein. The urease emerged as a sharp peak when eluted with 30ml of 50mM-phosphate buffer, pH6.8, containing 10mM-dithiothreitol. The enzyme in the eluate was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation. The precipitated enzyme was collected by centrifugation at 25000g for 20min and dissolved in a small volume of 0.1M-Tris/HCl buffer, pH8.0, containing 50mM-dithiothreitol, and stored frozen at -20°C . The enzyme was stable for more than 4 months and was used for all subsequent work.

Determination of molecular weight by gel electrophoresis. The principle of determining molecular weight of proteins by electrophoresis in polyacrylamide gels of two different concentrations (7.5% and 5%) was described by Zwaan (1967). In their system, because the electrophoresis was done at the one pH, the relative mobilities of proteins in the two gels were independent of the net charge, but were dependent on molecular weight and molecular shape. For globular proteins, the relative migration in the two gels was related to molecular weight. Fishbein *et al.* (1970) have used this procedure in the determination of the molecular weights of different isoenzymes of jack-bean urease. The procedures used here for preparing the gels (5% and 7.5% acrylamide with 5% bisacrylamide cross-linkage) and for the electrophoresis were the same as described by Davis (1964). Standard proteins (catalase, enolase and aldolase, 50µg each) and purified rumen urease (114µg) in 50µl of 0.1M-Tris/HCl buffer, pH8.0, containing 50mM-dithiothreitol, were each applied to separate gels (0.6cm×7cm). Electrophoresis was done at pH8.3 and 2.5mA/gel for 2h at 4°C . Proteins were stained with Amido Black and destained electrophoretically (Davis, 1964). Urease was located on the gels by the enzymic stain described by Blattler *et al.* (1967) as modified by Mahadevan *et al.* (1976). From the distance of migration of standard proteins and of urease in the two gels, the molecular weight of urease was determined by the graphical method described by Fishbein *et al.* (1970).

Determination of the molecular weight by Sephadex G-200 filtration. Sephadex G-200 was equilibrated for 72h in 50mM-potassium phosphate buffer, pH7.6, containing 10mM-dithiothreitol, and made into a column (2.6cm×34.5cm). The void volume (V_0)

determined with Blue Dextran as marker, was 53.0ml. The column was calibrated by using cytochrome *c* (13500), thyroglobulin (670000), serum albumin (67000), γ -globulin (160000) and myoglobin (16890) (12.5mg each in 1.0ml of the starting buffer). The urease preparation used contained 2.2mg of protein in the same buffer. Protein elution was monitored by the $A_{257.3}$. Flow rate was 7.7ml/h and fractions (5ml) were collected. Urease was assayed in 100µl portions of the eluate. The molecular weights of standard proteins were those given by Darnall & Klotz (1975). From the elution volumes, molecular weights were determined as described by Andrews (1964).

Determination of the molecular weight by sucrose-density-gradient centrifugation. The procedure was essentially the same as described by Martin & Ames (1961). A linear 5–20% (w/v) sucrose gradient in 0.05M-Tris/HCl buffer was formed in 2.54cm×8.89cm polyallomer tubes by using a Beckman linear-gradient former. Cytochrome *c* and thyroglobulin (200µg each in 200µl of 0.05M-Tris/HCl buffer, pH8.0) were layered on individual gradients. Total volume of the gradient was 34.8ml.

For the sedimentation experiments, the $(\text{NH}_4)_2\text{SO}_4$ present in the urease solution was removed by repeated ultrafiltration (Amicon apparatus with XM-50 membrane). The resulting concentrated urease was finally diluted with 50mM-Tris/HCl buffer, pH8.0, containing 10mM-dithiothreitol to give 2mg of protein/ml, and 200µl of this was layered on the gradient. The sucrose density gradient for urease was prepared as described above, but the sucrose solutions contained 10mM-dithiothreitol. Gradient centrifugation, in the absence of dithiothreitol from the sucrose, resulted in complete loss of urease activity.

The tubes (two for each protein) were centrifuged at 83000g (r_{av} 11.66cm) in a Beckman model L-2 ultracentrifuge with a SW27 swinging-bucket rotor for 21h at 2°C . The deceleration was done without brakes. The gradients were pumped out from the bottom and fractions (1.0ml) were collected. Standard proteins were monitored in the fractions by measuring the A_{280} by using a 1cm×2mm quartz cell and a Carl Zeiss PMQ II spectrophotometer. Urease in the fractions was assayed enzymically (see below).

The distances travelled from the meniscus to the centre of the fraction showing either maximum absorbance or maximum urease activity were measured. The molecular weight of urease was estimated by using the formula described by Martin & Ames (1961).

Assay of urease activity by using [^{14}C]urea. The procedure was the same as described by Mahadevan *et al.* (1976), except that the reaction mixtures contained 200µmol of Tris/HCl buffer, pH8.0, instead

of the phosphate buffer. All incubations were done in duplicate and the results are given as the average of two determinations. Duplicates agreed within 1–2%.

Assay for urea amidolyase by using [^{14}C]urea. The conditions of the assay were the same as described by Roon & Levenberg (1972). The techniques for the absorption and measurement of the liberated $^{14}\text{CO}_2$ were the same as for the urease assay.

Protein determination. All enzyme samples were dialysed against water for 20 h to remove dithiothreitol. Protein in the dialysed samples was determined by the method of Miller (1959), with bovine serum albumin as a standard.

Results and Discussion

Absence of urea amidolyase activity from crude extracts of rumen bacteria

The splitting of urea to CO_2 and NH_3 in certain micro-organisms is accomplished not by the action of urease, but by a complex of enzymes generally referred to as urea amidolyase (Roon & Levenberg, 1972). The reactions involve an ATP-dependent carboxylation of urea to allophanic acid, which is subsequently degraded to NH_3 and CO_2 . Since the assay system used in the present work involves the measurement of $^{14}\text{CO}_2$ formed from [^{14}C]urea, it was important to establish that the activity being measured was due to urease and not to urea amidolyase. In the present study, no requirement for ATP, Mg^{2+} or bicarbonate could be demonstrated for the release of $^{14}\text{CO}_2$ from [^{14}C]urea by the crude extracts of rumen bacteria.

Stability and solubilization of urease

The necessity for having sufficient concentration of dithiothreitol during storage of washed rumen-bacterial suspensions and during the solubilization of urease by ultrasonic disruption of the cells has been stated (Mahadevan *et al.*, 1976). In the absence of dithiothreitol, rumen urease was very unstable, like other microbial ureases (Larson & Kallio, 1954).

Purification of rumen urease

The results of a typical purification procedure are given in Table 1. Compared with the original bacterial suspension, about 60-fold purification was achieved. The yield of the purified enzyme, based on the amount present in the cell-free extract, was nearly 60%. Attempts at further purification by affinity chromatography as described by Wong & Shobe (1974) were not successful.

Purity of urease

Polyacrylamide-gel electrophoresis in 5% or 7.5% gels (see under 'Methods'), followed by staining for protein with Amido Black, revealed urease as the major protein. Densitometric tracings of the gel are

Table 1. *Purification of rumen urease*

See the text for details of purification procedure. Specific activity is expressed as μmol of urea hydrolysed/h per mg of protein.

Fraction	Specific activity	Activity (% of total)
Washed bacterial fraction	60	100
Cell-free extract	57	39
40–80%-satd.-(NH_4) $_2\text{SO}_4$ fraction	354	45
Calcium phosphate-gel eluate	419	32
Hydroxyapatite column	3595	24

shown in Fig. 1. Integration of the area under the curve indicated that the urease was approx. 80% pure. Assuming a maximum of 20% contamination, the maximum specific activity of pure rumen urease might be expected to be in the range of 4–5 mmol of urea hydrolysed/h per mg of protein. This represents about 600–800-fold increase over the activity of urease in the isolated ureolytic strain of *Selenomonas ruminantium* described by John *et al.* (1974).

Enzymic localization of urease on polyacrylamide gels

The crude extract or the enzyme at various stages of purification was subjected to polyacrylamide-gel electrophoresis as described by Davis (1964) and the gels were subsequently stained for urease activity as described under 'Methods'. With either crude extract or with purified enzyme only one urease-positive band was present. No other urease-positive bands were observed with changes in gel composition or pH. For the purified enzyme, the protein band stained with Amido Black coincided exactly with the urease band (Fig. 1).

Molecular weight of purified rumen urease

The molecular weight determined (a) by electrophoresis on 7.5% and 5% polyacrylamide gel was 135 000, (b) by sucrose-density-gradient centrifugation was 130 000 and (c) by Sephadex G-200 filtration was 125 000. Therefore the molecular weight of rumen urease is about 130 000, which is similar to that of *P. mirabilis* urease (Andersen *et al.*, 1969), but is about one-quarter the size of α -urease from jack bean (Fishbein *et al.*, 1975). The smallest active species of jack-bean urease so far identified has a molecular weight of 240 000 (Fishbein, 1969). The retention of full enzyme activity by microbial ureases having molecular weights in the range of 130 000 possibly indicates a fundamental difference in the molecular structure of urease from plant and microbial sources.

Absence of multiple forms of ureases

It is surprising that in spite of the large number of ureolytic organisms identified in the rumen (Slyter

Fig. 1. Densitometric tracing of the polyacrylamide gel stained for protein

Purified rumen urease (114 μ g of protein) was subjected to electrophoresis, stained for protein with Amido Black, and destained as described in the text. Tracings were made with a Joyce-Loebl Chromoscan densitometer. Tracking dye (a) and urease (b).

et al., 1968; Allison, 1970), urease with the same electrophoretic mobility and molecular weight is produced. In this connexion, the observations of Cook (1976) about the mechanism of production of urease in rumen microbes are significant. Cook (1976) has suggested that the production of urease in *Streptococcus faecium*, isolated from sheep rumen, is controlled by a plasmid gene. The transfer of plasmid genes from one organism to others, when grown in mixed cultures, has been demonstrated (Lacey, 1975). It appears possible therefore that such a transfer might occur in rumen micro-organisms growing as a mixed culture, resulting in the production of identical urease enzymes by the different organisms.

Effect of pH

The purified rumen urease exhibited a sharp pH optimum at pH 8.0 in Tris/HCl buffer. With the same substrate concentration of 125 mM-urea, the activity at pH 7.0 was 52% and at pH 8.5 was only 42% of that at the optimum pH. Brent *et al.* (1971) in their studies on bovine rumen urease assayed the activity at pH 6.8, whereas Baintner (1964) reported the pH optimum of sheep rumen urease activity as 7.6. In contrast with the rumen urease, the urease from *P. mirabilis* has been reported to be maximally active over the pH range 6–8.3 (Andersen *et al.*, 1969). Jack-bean urease exhibits different pH optima depending on the type of buffer used (Reithel, 1971).

Effect of phosphate ion

Phosphate ion is known to inhibit jack-bean urease (Reithel, 1971). However, when increasing amounts of phosphate were added to the reaction mixture containing Tris/HCl buffer, there was no inhibition of rumen urease.

Effect of substrate concentration

The effect of increasing amounts of urea on the velocity of the hydrolysis of urea was determined in three separate experiments. From these data the K_m and V_{max} were determined by using the computer program described by Cleland (1963). The V_{max} was 3.2 ± 0.25 (mean $\pm$ S.E.M.) mmol of urea hydrolysed/h per mg, and the K_m for urea was $8.3 \times 10^{-4} \pm 1.7 \times 10^{-4}$ M (mean $\pm$ S.E.M.). Brent *et al.* (1971) reported K_m of 3.6×10^{-3} M for partially purified bovine rumen urease, whereas Baintner (1964) obtained K_m values ranging from 2×10^{-3} M to 2×10^{-4} M with urease from sheep rumen. Jack-bean urease has K_m 3.28×10^{-3} M (Blakeley *et al.*, 1969).

Effect of dithiothreitol

The necessity for dithiothreitol throughout the purification procedure and storage has been indicated above. There was no additional requirement for dithiothreitol in the assay.

Effect of EDTA

Dixon *et al.* (1975b) consider jack-bean urease to be a metalloenzyme. Therefore the effect of EDTA on rumen urease was investigated. With increasing concentration of EDTA, rumen urease was not inhibited but instead showed about 20% stimulation. The stimulation might be the result of removal of traces of contaminating inhibitory heavy metals. Dixon *et al.* (1976) have subsequently drawn attention to the fact that many metalloenzymes are not inhibited by EDTA. Therefore the absence of inhibition by high concentrations of EDTA under the conditions used should not be taken to indicate that rumen urease is not a metalloenzyme.

Effect of metal ions

Jones *et al.* (1964) concluded that Mn^{2+} , Mg^{2+} , Ca^{2+} , Ba^{2+} and Sr^{2+} , up to 20mM, stimulated rumen urease activity in intact bacterial cells, extracts of acetone-dried bacteria or sonically disrupted bacterial cells. Because of the economic importance of inhibiting rumen bacterial urease activity (Mahade-

van *et al.*, 1976), the effect of metal ions on purified rumen urease was reinvestigated. The results (Table 2) show that in contrast with the results of Jones *et al.* (1964), Mn^{2+} and Ba^{2+} strongly inhibited the urease, whereas Mg^{2+} , Ca^{2+} and Sr^{2+} had little or no effect on the enzyme. None of the metal ions activated rumen urease.

Hughes *et al.* (1969) listed the effectiveness of heavy-metal ions as inhibitors of jack-bean urease in the order $Hg^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Co^{2+} > Ni^{2+}$. In the present study (Table 2) the order of effectiveness of these metals as inhibitors of rumen urease was found to be $Cu^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} > Cd^{2+} > Hg^{2+}$. Hughes *et al.* (1969) considered the mechanism of inhibition of urease by these metals as being due to blocking of essential thiol groups on the enzyme. In addition, Dixon *et al.* (1975b) have suggested that heavy-metal ions might replace some essential trace elements in enzymes, resulting in the formation of inactive forms of enzymes.

Effect of thiol reagents

The enzyme was inhibited by *p*-chloromercuribenzenesulphonate and *N*-ethylmaleimide (Table 3), indicating that thiol groups on the enzyme are essential for activity. In this respect, rumen urease resembles jack-bean urease (Reithel, 1971) and other bacterial ureases (Larson & Kallio, 1954; Andersen *et al.*, 1969).

Effect of hydroxyurea

Fishbein *et al.* (1965) demonstrated that hydroxyurea is a substrate for jack-bean urease and that the rate of hydrolysis of hydroxyurea was less than one-hundredth the rate of hydrolysis of urea. In the present study, hydroxyurea was incubated with purified rumen urease under the same conditions as described for urea and the hydroxylamine produced was measured as described by Fishbein *et al.* (1965). The rate of hydrolysis of hydroxyurea was 200-

Table 2. *Effect of metal ions on purified rumen urease*

The chlorides of the metals were added to the reaction mixture. The rest of the procedure was the same as described in the text. Activity without added metal ions was taken as 0% inhibition.

Metal ion	Final concn. (mM)	Inhibition of activity (%)
Mn^{2+}	2	75
	5	93
	10	97
Mg^{2+}	2	0
	5	3
	10	20
Ca^{2+}	2	0
	5	0
	10	5
Sr^{2+}	2	0
	5	0
	10	0
Ba^{2+}	2	18
	5	35
	10	55
Hg^{2+}	0.02	12
	0.2	100
Cu^{2+}	0.02	95
	0.2	100
Zn^{2+}	0.02	93
	0.2	100
Cd^{2+}	0.02	20
	0.2	100
Ni^{2+}	0.02	84
	0.2	97
Co^{2+}	0.02	90
	0.2	97

Table 3. *Effect of thiol reagents on purified rumen urease*

All components of the reaction mixture, without substrate, were incubated at 22°C for 10 min in a total volume of 1.5 ml. After 10 min substrate (0.5 ml) was added. The rest of the procedure was the same as described in the text. Control activity, without inhibitors, was taken as 0% inhibition.

Reagent	Final concn. (mM)	Inhibition of activity (%)
<i>p</i> -Chloromercuribenzenesulphonate	0.01	18.7
	0.1	100.0
<i>N</i> -Ethylmaleimide	0.01	26.3
	0.1	96.7
	1.0	100.0

Table 4. *Inhibition of purified rumen urease by hydroxyurea and the reversal of inhibition by urea*

The enzyme, hydroxyurea and buffer were incubated at room temperature (22°C) for 10 min in a total volume of 1.5 ml before the indicated amount of urea (0.5 ml) was added. The rest of the procedure was the same as described in the text.

Hydroxyurea in 2.0 ml reaction (μ mol)	Urea in 2.0 ml reaction (μ mol)	Urea hydrolysed (μ mol/h per mg of protein)
0	100	3380
100	100	1380
100	200	1830
100	300	2090
100	400	2490
100	500	2650

400 μ mol/h per mg, which was about one-eighth to one-sixteenth the rate of hydrolysis of urea.

Hydroxyurea inhibited the hydrolysis of urea (Table 4). The inhibition was reversed by increasing the concentration of urea. In this respect, rumen urease differs from jack-bean urease, which is irreversibly inhibited by hydroxyurea (Fishbein & Carbone, 1965).

Inhibition by acetohydroxamate

Although Baintner (1964) considered sheep rumen urease to be inhibited competitively, Brent *et al.* (1971) concluded that bovine rumen urease was inhibited non-competitively and reversibly by acetohydroxamate. In the present study, the results in Table 5 indicate that the inhibition of rumen urease by acetohydroxamate was reversed by increasing urea concentration.

The mechanism of inhibition of urease by acylhydroxamates is not understood (Fishbein & Carbone, 1965; Blakeley *et al.*, 1969), apart from the fact that a CONH(OH) grouping is essential for inhibition (Kobashi *et al.*, 1962). Dixon *et al.* (1975a) suggested that the inhibition of urease by hydroxamates is indicative of the metalloenzyme nature of urease.

When urea is fed to ruminants it is rapidly hydrolysed by rumen urease. This rapid rate of urea hydrolysis results in losses of NH_3 . In the past, different approaches have been taken to control and decrease the activity of urease in the rumen in order to increase the efficiency of utilization of dietary urea by the ruminant (Allison, 1970). In those studies, it has been assumed that rumen urease has the same properties as jack-bean urease. The present study shows that rumen urease is different in many respects from jack-bean urease. These facts have to be taken into consideration in future studies on the mechanisms controlling the production and activity of urease *in*

Table 5. *Effect of increasing concentration of urea on acetohydroxamate-inhibited rumen urease*

The assay of urease without the inhibitor was done as described in the text with increasing amounts of urea in the reaction mixture. When assayed in the presence of the inhibitor, before the addition of urea, the enzyme was mixed with buffer and acetohydroxamate to give a final concentration of 0.2 mM acetohydroxamate.

Urea in 2.0 ml reaction mixture (μ mol)	Urea hydrolysed (μ mol/h per mg of protein)	
	–inhibitor	+inhibitor
2	650	70
3	890	100
4	1040	130
20	910	340
100	1040	540

in vivo and in devising practical methods of decreasing the rate of production of NH_3 from urea in the rumen.

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References

- Allison, M. J. (1970) in *Physiology of Digestion and Metabolism in the Ruminant* (Phillipson, A. T., ed.), pp. 461–463, Oriel Press, Newcastle upon Tyne
- Andersen, J. A., Kopko, F., Siedler, A. J. & Nohle, E. G. (1969) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **28**, 764
- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
- Baintner, K., Jr. (1964) *Allatenyesties* **13**, 17–25
- Blakeley, R., Webb, E. C. & Zerner, B. (1969) *Biochemistry* **8**, 1984–1990
- Blattler, D. P., Contaix, C. C. & Reithel, F. J. (1967) *Nature (London)* **216**, 274–275
- Brent, B. E., Adepoju, A. & Portella, F. (1971) *J. Anim. Sci.* **32**, 794–798
- Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 188–196
- Cook, A. R. (1976) *J. Gen. Microbiol.* **92**, 49–58
- Darnall, D. W. & Klotz, I. (1975) *Arch. Biochem. Biophys.* **166**, 651–682
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dixon, N. E., Gazzola, C., Watters, J. J., Blakeley, R. & Zerner, B. (1975a) *J. Am. Chem. Soc.* **97**, 4130–4131
- Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B. (1975b) *J. Am. Chem. Soc.* **97**, 4131–4133
- Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B. (1976) *Science* **191**, 1144–1150
- Fishbein, W. N. (1969) *Ann. N.Y. Acad. Sci.* **147**, 857–881
- Fishbein, W. N. & Carbone, P. P. (1965) *J. Biol. Chem.* **240**, 2407–2414
- Fishbein, W. N., Winter, T. S. & Davidson, J. D. (1965) *J. Biol. Chem.* **240**, 2402–2406
- Fishbein, W. N., Nagarajan, K. & Scurzi, W. (1970) *J. Biol. Chem.* **245**, 5985–5992

- Fishbein, W. N., Nagarajan, K. & Scurzi, W. (1975) in *Isozymes I: Molecular Structure* (Markert, C. L., ed.), pp. 403–417, Academic Press, New York
- Hughes, R. B., Katz, S. A. & Stubbins, S. E. (1969) *Enzymologia* **36**, 332–334
- John, A. H., Issacson, H. R. & Bryant, M. P. (1974) *J. Dairy Sci.* **57**, 1003–1014
- Jones, G. A., MacLeod, R. A. & Blackwood, A. C. (1964) *Can. J. Microbiol.* **10**, 379–387
- Kobashi, K., Hase, J. & Uehara, K. (1962) *Biochim. Biophys. Acta* **65**, 380–383
- Lacey, R. W. (1975) *Bacteriol. Rev.* **39**, 1–32
- Larson, A. D. & Kallio, R. E. (1954) *J. Bacteriol.* **68**, 67–73
- Mahadevan, S., Sauer, F. D. & Erfle, J. D. (1976) *J. Anim. Sci.* **42**, 745–753
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Miller, G. L. (1959) *Anal. Chem.* **31**, 964
- Pearson, R. M. & Smith, J. A. B. (1943) *Biochem. J.* **37**, 148–153
- Reithel, F. J. (1971) *Enzymes 3rd Ed.* **4**, 1–21
- Roon, R. J. & Levenberg, B. (1972) *J. Biol. Chem.* **247**, 4107–4113
- Seneca, H., Peer, P. & Nally, R. (1962) *Nature (London)* **193**, 1106–1107
- Slyter, L. L., Oltjen, R. R., Kern, D. L. & Weaver, J. M. (1968) *J. Nutr.* **94**, 185–191
- Wong, B. L. & Shobe, C. R. (1974) *Can. J. Microbiol.* **20**, 623–630
- Zwaan, J. (1967) *Anal. Biochem.* **21**, 155–168