Biochem. J. (1965) **94**, 384

Organ Specificity and Lactate-Dehydrogenase Activity

DIFFERENTIAL INHIBITION BY UREA AND RELATED COMPOUNDS

BY WENDY A. WITHYCOMBE, D. T. PLUMMER* AND J. H. WILKINSON
Department of Chemical Pathology, Westminster Medical School (University of London), London, S.W. 1

(*Received* 6 *May* 1964)

1. The effect of urea on the lactate-dehydrogenase activities of human-heart and -liver tissue extracts and on crystalline ox-heart and rabbit-muscle enzyme have been determined. Similar studies on electrophoretically separated isoenzyme fractions have shown an inverse relationship between sensitivity to urea inhibition and electrophoretic mobility. 2. With pyruvate as substrate a sharp change in the nature of the inhibition of tissue lactate dehydrogenase with increasing concentrations of urea occurs at 1 M or 4 M with the electrophoretically slow and fast isoenzymes respectively. 3. At concentrations of urea less than 1 M, inhibition of the purified enzymes is competitive with respect to pyruvate and 2-oxobutyrate. 4. Similar studies have been carried out with methylurea and hydantoic acid, both of which are more potent inhibitors than urea.

Although lactate dehydrogenase catalyses the reversible oxidation of lactate irrespective of its origin, each tissue enzyme possesses characteristic physicochemical properties. These variations have now been shown to be due to the occurrence in different tissues of five distinct but related isoenzymes that can be separated electrophoretically (Wieland & Pfleiderer, 1957; Vesell & Bearn, 1957). Among the properties that may be used for distinguishing isoenzymes are the effects of inhibitors. Wieland, Pfleiderer & Ortanderl (1959) demonstrated that sulphite preferentially inhibits the anodic fractions, LD_1 and LD_2, and Plummer & Wilkinson (1961, 1963) found that oxalate had a marked inhibitory effect on LD_1 and LD_2, but had relatively little action on the slow-moving components, LD_4 and LD_5.

Since lactate-dehydrogenase isoenzymes are tetramers that are disrupted into inactive monomers by treatment with 12 M-urea or 5 M-guanidine hydrochloride (Appella & Markert, 1961, 1962; Cahn, Kaplan, Levine & Zwilling, 1962), we investigated the effects of lower concentrations of urea on the activities of human-heart and -liver preparations and on the purified rabbit-muscle and ox-heart enzymes. Most of this work has been carried out with pyruvate and 2-oxobutyrate as substrates, but the oxidation of lactate and 2-hydroxybutyrate has also been investigated. A preliminary account was presented at a meeting of the Biochemical Society (Plummer, Wilkinson & Withycombe, 1963*b*).

* Present address: Department of Physiology, Chelsea College of Science and Technology, London, S.W. 3.

MATERIALS AND METHODS

Enzymes. Crystalline rabbit-muscle and ox-heart lactate dehydrogenase were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and the Worthington Biochemical Corp., Freehold, N.J., U.S.A., respectively.

Tissue extracts. Extracts of human heart, kidney and liver were prepared by the method of Plummer, Elliott, Cooke & Wilkinson (1963*a*).

Determination of enzyme activity. (*a*) With $NADH_2$. A spectrophotometric method based on that of Wróblewski & LaDue (1955) was used. The reaction was carried out in Sørensen 0·067 M-phosphate buffer, pH 7·4, containing $NADH_2$ (0·35 μmole, freshly prepared) and final substrate concentrations, unless otherwise stated, of 0·7 mM-sodium pyruvate or 3·3 mM-sodium 2-oxobutyrate (Rosalki & Wilkinson, 1960). For inhibition studies various amounts of inhibitor were incorporated into the mixture to produce the final concentrations quoted. Enzyme–inhibitor mixtures were allowed to stand at 25° for 15 min. in all cases before determination of enzyme activity. Extinction measurements were made at 25° and 340 mμ in a Unicam SP.500 spectrophotometer. Activities were expressed as μmoles of $NADH_2$ oxidized/min.

(*b*) With NAD. Dehydrogenase activity was estimated at pH 8·8 in 1 M-tris buffer (Wacker & Dorfman, 1962) with 5·2 mM-NAD and final substrate concentrations of 80 mM-sodium lactate or 80 mM-sodium DL-2-hydroxybutyrate. Activities were expressed as μmoles of NAD reduced/min.

Starch-block electrophoresis. This was carried out as described by Plummer *et al.* (1963*a*).

Cellulose acetate electrophoresis. This was carried out in a Shandon Universal apparatus (Kohn, 1960). Lactate-dehydrogenase isoenzymes were located by the tetrazolium staining technique of Barnett (1962), or the cellulose acetate strips were cut into 3 mm. sections, each of which was eluted with Sørensen phosphate buffer, pH 7·4, and the activity of the eluate determined as described above.

RESULTS

Inhibition by urea. The effects of urea on the activities of purified ox-heart and rabbit-muscle lactate dehydrogenases and on those of human-heart and human-liver preparations were investigated. With pyruvate as substrate 2 M-urea completely inhibited the activity of the liver preparation and that of crystalline rabbit-muscle lactate dehydrogenase. At the same urea concentration crystalline ox-heart enzyme and the human-heart extract were inhibited by only about 20%, but were almost completely inactivated by 6 M-urea. The inhibitory effect was greater in all cases when 2-oxobutyrate was used as substrate. The liver preparation and the rabbit-muscle enzyme were completely inactivated by 1·5 M-urea, and the ox-heart enzyme by 4·0 M-urea. When the degree of inhibition was expressed as v/v_i, where v represents the activity in the absence of the inhibitor and v_i the activity in its presence, and was plotted against inhibitor concentration, similar types of response were obtained regardless of the enzyme source or the substrate (Figs. 1 and 2). With increasing urea concentration there was at first a slow increase in the inhibitory effect until a critical point was reached at which a sharp inflexion in the curve occurred and inhibition became virtually complete. The concentration at which this occurred varied according to the source of the enzyme.

Similar experiments were conducted on the partially purified isoenzyme fractions of human-heart,

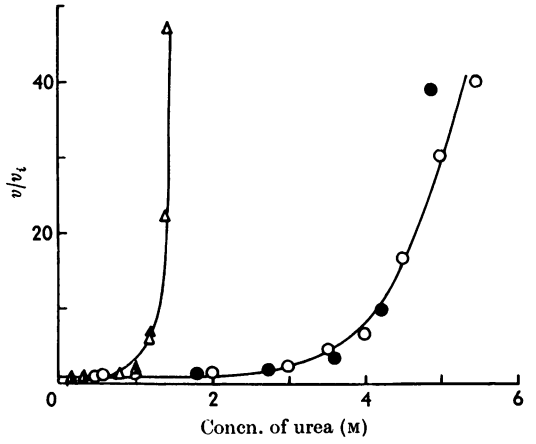

Fig. 1. Effect of urea on lactate-dehydrogenase activities of preparations of human heart (○) and human liver (△) and of crystalline ox-heart (●) and rabbit-muscle (▲) enzymes with 0·7 mM-pyruvate as substrate. v/v_i, Activity in the absence of urea/activity in the presence of urea.

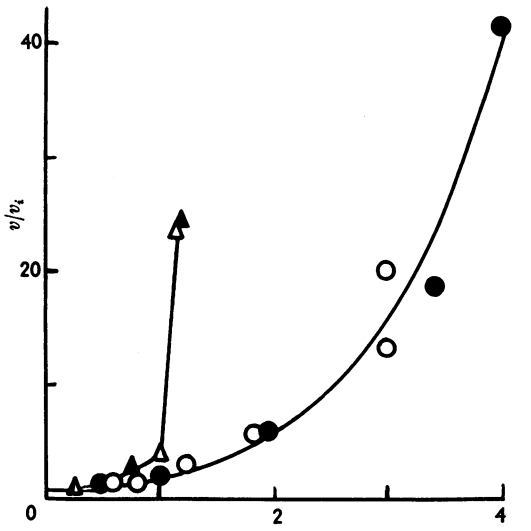

Fig. 2. Effect of urea on lactate-dehydrogenase activities of preparations of human heart (○) and human liver (△) and of crystalline ox-heart (●) and rabbit-muscle (▲) enzymes with 3·3 mM-2-oxobutyrate as substrate. v/v_i, Activity in the absence of urea/activity in the presence of urea.

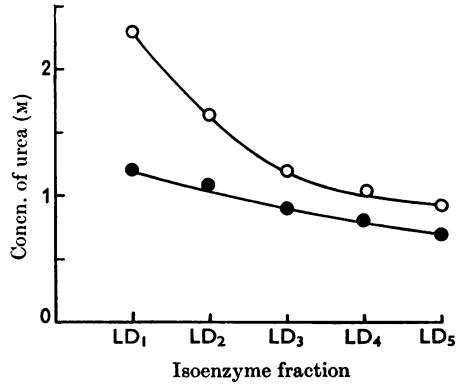

Fig. 3. Urea concentration giving 50% inactivation ($v/v_i = 2·00$) of tissue lactate-dehydrogenase isoenzymes of human-heart and -kidney extracts (LD_1, LD_2 and LD_3) and human-liver extract (LD_4 and LD_5) with 0·7 mM-pyruvate (○) and 3·3 mM-2-oxobutyrate (●) as substrates.

-kidney and -liver extracts. Isoenzymes were separated by starch-block or cellulose acetate electrophoresis and each fraction was treated with urea. Similar results were obtained but the fast-moving isoenzymes were more resistant to inactivation by urea than were the slow-moving ones. The inflexion in the curve occurred in all cases at a point where there was a decrease in activity of approx. 50%. The urea concentration at which this occurred decreased from LD_1 to LD_5 (Fig. 3).

Urea exerted a similar effect on the reverse reaction, but with the crystalline enzymes little difference was observed between the curves obtained with lactate as substrate and those obtained with 2-hydroxybutyrate (Fig. 4). When the crystalline ox-heart enzyme was used with lactate as substrate the point of inflexion in the curve occurred at a urea concentration of 2 M, whereas with pyruvate 4 M-urea was required to produce the same degree of inactivation. No such difference was found between the sensitivities of this enzyme to urea with 2-hydroxybutyrate or 2-oxobutyrate as substrate. With both keto and hydroxy acids, the rabbit-muscle enzyme gave similar results.

Combination with the coenzyme did not protect the enzyme against inhibition by urea since the same results were observed when NAD (or $NADH_2$) was added to the reaction mixture 1 hr. before the urea as when it was added immediately afterwards. This was found with both low and high concentrations of urea, and when excess of the coenzyme was used.

Effect of substrate concentration on urea-treated enzyme. Effects of variation in the substrate concentration on the rate of reduction of pyruvate at two different urea concentrations are shown as Lineweaver & Burk (1934) reciprocal plots of the initial reaction velocity (Fig. 5). Ox-heart enzyme gave apparent enzyme–substrate constants 0·065 mM in the absence of urea, 0·107 mM with 1 M-urea, and 1·00 mM with 3 M-urea. Parallel experiments with 2-oxobutyrate as substrate showed that urea apparently competitively inhibited the reduction of this substrate more effectively than it did the reduction of pyruvate. Similar effects observed with the rabbit-muscle enzyme are shown in Fig. 6.

Time-dependence of urea inhibition. At urea concentrations less than 1 M, the inhibitory effect on rabbit-muscle lactate dehydrogenase was independent of the period of exposure to urea (0–6 hr.), but at higher concentrations the degree of inhibition increased with time. With the ox-heart enzyme, inhibition was independent of time at concentrations up to 2 M, and activity was partly restored when

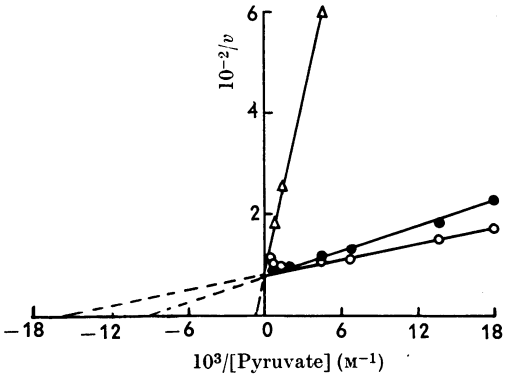

Fig. 5. Reciprocal plots showing effects of variation in pyruvate concentration on lactate-dehydrogenase activity of ox-heart enzyme in the absence (○) and presence of 1·0 M-urea (●) and 3·0 M-urea (△).

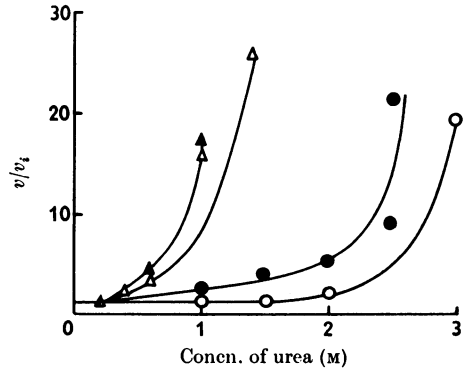

Fig. 4. Effect of urea on oxidation of lactate (○, △) and 2-hydroxybutyrate (●, ▲) by crystalline ox-heart (○, ●) and rabbit-muscle (△, ▲) enzymes. v/v_i, Activity in the absence of urea/activity in the presence of urea.

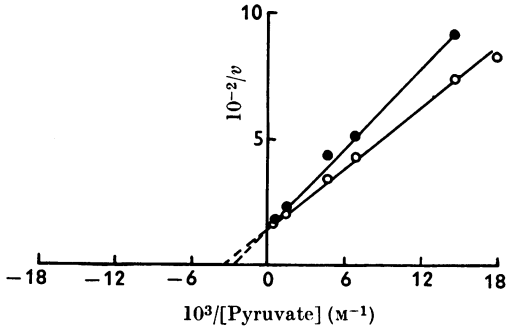

Fig. 6. Reciprocal plots showing effect of variation in pyruvate concentration on lactate-dehydrogenase activities of rabbit-muscle enzyme in the absence (○) and presence of 0·6 M-urea (●).

the urea was removed by overnight dialysis against phosphate buffer at 0°. Inhibition was time-dependent at higher concentrations (Fig. 7), and no recovery of activity was observed when solutions were subsequently dialysed against phosphate buffer or 3 M-sodium chloride, nor when the urea concentration was decreased to a sub-inhibitory level by dilution. Similar results were obtained with the rabbit-muscle enzyme, but inhibition appeared to be irreversible at concentrations of urea as low as 0·6 M.

Related urea compounds. The effects of three compounds, methylurea, hydantoic acid and cyanate, chemically related to urea, were also investigated. The inhibitory effect of methylurea on ox-heart lactate dehydrogenase was much stronger than that of urea, for a concentration of 0·1 M caused the sharp inflexion in the inhibition plots (Fig. 8) with both pyruvate and 2-oxobutyrate. A similar pattern of inhibition was observed with lactate and 2-hydroxybutyrate as substrates. With the rabbit-muscle enzyme and either pyruvate or 2-oxobutyrate as substrate a direct relationship between the degree of inhibition and the concentration of methylurea was observed (Fig. 9).

Hydantoic acid did not distinguish between LD_1 and LD_5 to the same degree as urea, although it did inhibit the slow isoenzymes of rabbit-muscle enzyme slightly more than it did the fast isoenzymes of ox-heart lactate dehydrogenase. Methylurea, on the other hand, appeared to have slightly more effect on LD_1 than on LD_5, since a concentration of 0·2 M caused 30% inactivation of the crystalline rabbit-muscle enzyme and 45% inactivation of the ox-heart enzyme with pyruvate as substrate. Very similar values were obtained with 2-oxobutyrate as substrate.

Methylurea competitively inhibited the reduction of 2-oxobutyrate by both ox-heart and rabbit-muscle enzymes to a greater extent than that of pyruvate. Reciprocal plots gave different values for the apparent enzyme–substrate constants for the enzyme treated with the inhibitor and the untreated enzyme (Table 1).

Similar plots for hydantoic acid showed it also to be a competitive type of inhibition. When the degree of inactivation was expressed as v/v_i there was a linear increase with the concentration of hydantoate, the effect being greater with 2-oxobutyrate than with pyruvate as substrate.

These studies were extended to the effect of cyanate on lactate-dehydrogenase activity. Preliminary studies on the reduction reaction indicated that cyanate caused considerable inhibition. Rather

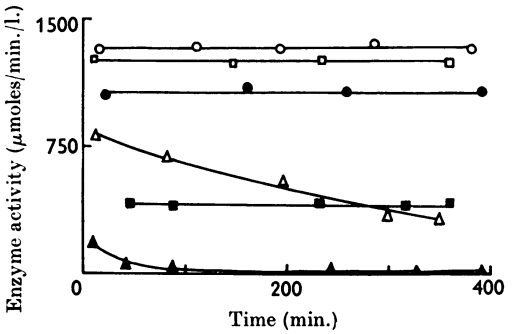

Fig. 7. Effect of time on the rates of reduction of pyruvate (○, △, □) and 2-oxobutyrate (●, ▲, ■) by ox-heart enzyme untreated (○, ●) or treated with 3 M-urea (△, ▲) or 1 M-urea (□, ■).

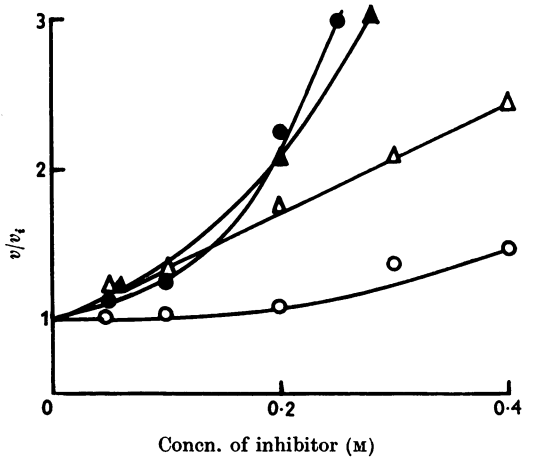

Fig. 8. Effect of hydantoate (○, △) and methylurea (●, ▲) on ox-heart lactate-dehydrogenase activity with 0·7 mM-pyruvate (○, ●) and 3·3 mM-2-oxobutyrate (△, ▲) as substrates.

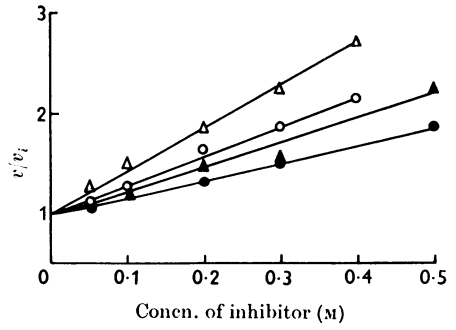

Fig. 9. Effect of hydantoate (○, △) and methylurea (●, ▲) on rabbit-muscle lactate-dehydrogenase activity with 0·7 mM-pyruvate (○, ●) and 3·3 mM-2-oxobutyrate (△, ▲) as substrates.

Table 1. *Results of Lineweaver–Burk reciprocal plots showing the effect of methylurea and hydantoic acid on the apparent substrate constants, K_m, for pyruvate and 2-oxobutyrate of crystalline ox-heart and rabbit-muscle lactate dehydrogenases*

	Substrate ...	... Pyruvate		2-Oxobutyrate	
Enzyme source	Inhibitor	K_m (mM)	$V_{max.}$ (μmoles/min./mg. of protein)	K_m (mM)	$V_{max.}$ (μmoles/min./mg. of protein)
Rabbit muscle	None	0·22	111	3·33	29
Rabbit muscle	0·2 M-Methylurea	0·32	111	3·84	29
Rabbit muscle	0·2 M-Hydantoate	0·59	90	5·57	29
Ox heart	None	0·073	24	0·83	91
Ox heart	0·1 M-Methylurea	0·059	24	1·09	99
Ox heart	0·2 M-Hydantoate	0·280	24	2·64	99

surprisingly it was found to activate the oxidation reaction, but subsequently this appeared to be due to the effect of an impurity in the cyanate that reduced NAD to $NADH_2$. Cyanate itself seems to have little effect on lactate-dehydrogenase activity.

DISCUSSION

When the inhibitory effect of urea on various enzyme preparations is expressed as v/v_i and plotted against the urea concentrations, the curve shows a sharp inflexion. There is an initial flat portion where inactivation is slight, then at a critical concentration, which varies with the enzyme source, the degree of inhibition increases rapidly. This phenomenon is observed with both pyruvate and 2-oxobutyrate and with the corresponding hydroxy acids. The curve is similar to that observed by Callaghan & Martin (1962, 1963), who studied the effect of urea on albumin and γ-globulin. They interpreted the sudden change as being due to rupture of hydrogen bonds and unfolding of the molecule, and it may be that the break in the curve of lactate-dehydrogenase activity indicates a similar conformational change of the enzyme protein. Appella & Markert (1961, 1962) report that 12 M-urea dissociates the lactate-dehydrogenase molecule into four sub-units of identical molecular weight. It seems probable that lower concentrations may cause loss of hydrogen-bonding and unfolding of the helices. Our failure to detect enzyme activity after removal of the urea parallels the experience of Markert & Appella (1963), who attribute the effect to destruction of the primary and secondary (helical) structure of the monomeric sub-units.

The more weakly bound substrate, 2-oxobutyrate, was more easily displaced from the enzyme than pyruvate, irrespective of the source, by all three inhibitors, urea, methylurea and hydantoate. Inhibition was competitive at low concentrations, but since the action of urea at high concentrations was time-dependent, it was not possible to determine accurately the type of inhibition, although it appears likely that cleavage of hydrogen bonds was occurring. An attempt was made to compare the effects of urea on certain physical properties of the enzyme with that on lactate-dehydrogenase activity, but the techniques used for measuring changes in extinction at 210 mμ or in refractive index were insufficiently sensitive to be informative.

Lactate dehydrogenase forms a ternary complex with the coenzyme and substrate molecules (Winer & Schwert, 1959; Novoa, Winer, Glaid & Schwert, 1959), and the inability of excess of coenzyme to protect the enzyme from the action of urea suggests that urea does not combine with the active centre of the enzyme molecule. On the other hand, the competitive nature of the inhibition at low concentrations suggests that urea can displace substrate from the active centres. It seems likely therefore that two different effects are exerted, and at high concentrations competitive displacement of substrate may be obscured by changes in the secondary or tertiary structure of the enzyme protein.

Whatever the mechanism involved, the inhibitory action of urea and certain of its derivatives appears to have potential value for the differentiation of lactate-dehydrogenase isoenzymes, and in this connexion reports by Richterich & Burger (1963) and Brody (1964) are of interest. These investigators also found that the electrophoretically slow isoenzymes, LD_4 and LD_5, from a number of tissues are preferentially inhibited by urea. By contrast, Plummer & Wilkinson (1961, 1963) found that oxalate selectively inhibits the fast isoenzymes.

Studies on sera from various pathological cases (P. M. Emerson, D. T. Plummer, J. H. Wilkinson & W. A. Withycombe, unpublished work) have shown that urea inhibition may be used as an additional aid in the diagnosis of myocardial infarction and liver disease. The slow-moving lactate-dehydrogenase isoenzymes, which are prevalent in the sera of patients with liver disease, are readily inactivated

by urea, whereas the activity of sera from patients with myocardial infarction is affected but little.

This work was supported by a grant from the Medical Research Council for scientific assistance and expenses (to J. H. W.), and also by a grant from the Endowment Funds of Westminster Hospital. Skilled technical assistance by Miss Margaret Cromwell-Thomas, kindly financed by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, is also gratefully acknowledged.

REFERENCES

Appella, E. & Markert, C. L. (1961). *Biochem. biophys. Res. Commun.* **6**, 171.
Appella, E. & Markert, C. L. (1962). *Fed. Proc.* **21**, 253.
Barnett, H. (1962). *Biochem. J.* **84**, 83P.
Brody, I. A. (1964). *Nature, Lond.*, **201**, 685.
Callaghan, P. & Martin, N. H. (1962). *Biochem. J.* **83**, 144.
Callaghan, P. & Martin, N. H. (1963). *Biochem. J.* **87**, 225.
Cahn, B. D., Kaplan, N. O., Levine, L. & Zwilling, E. (1962). *Science*, **136**, 962.
Kohn, J. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 2, p. 56. Ed. by Smith, I. London: W. Heinemann Medical Books Ltd.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Markert, C. L. & Appella, E. (1963). *Ann. N.Y. Acad. Sci.* **103**, 915.
Novoa, W. B., Winer, A. D., Glaid, A. J. & Schwert, G. W. (1959). *J. biol. Chem.* **234**, 1143.
Plummer, D. T., Elliott, B. A., Cooke, K. B. & Wilkinson, J. H. (1963*a*). *Biochem. J.* **87**, 416.
Plummer, D. T. & Wilkinson, J. H. (1961). *Biochem. J.* **81**, 38P.
Plummer, D. T. & Wilkinson, J. H. (1963). *Biochem. J.* **87**, 423.
Plummer, D. T., Wilkinson, J. H. & Withycombe, W. A. (1963*b*). *Biochem. J.* **89**, 48P.
Richterich, R. & Burger, A. (1963). *Helv. physiol. acta*, **21**, 54.
Rosalki, S. B. & Wilkinson, J. H. (1960). *Nature, Lond.*, **188**, 1110.
Vesell, E. S. & Bearn, A. G. (1957). *Proc. Soc. exp. Biol., N.Y.*, **94**, 96.
Wacker, W. E. C. & Dorfman, L. E. (1962). *J. Amer. med. Ass.* **181**, 972.
Wieland, T. & Pfleiderer, G. (1957). *Biochem. Z.* **329**, 112.
Wieland, T., Pfleiderer, G. & Ortanderl, F. (1959). *Biochem. Z.* **331**, 103.
Winer, A. D. & Schwert, G. W. (1959). *J. biol. Chem.* **234**, 1155.
Wróblewski, F. & LaDue, J. S. (1955). *Proc. Soc. exp. Biol., N.Y.*, **90**, 210.