Metabolism of D-Glucuronolactone in Mammalian Systems

INHIBITORY PROPERTIES OF THE PRODUCTS OF D-GLUCURONOLACTONE-DEHYDROGENASE ACTION

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1. D-Glucuronolactone was converted into D-glucaro-(1→4)-lactone, a β -glucuronidase inhibitor, probably via the intermediate D-glucaro-(1→4)-(6→3)-dilactone, by a dehydrogenase in human-liver extracts. 2. Similar experiments with mouse-liver extracts appeared to yield only D-glucaric acid or a non-inhibitory lactone. 3. A strong β -glucuronidase inhibitor was present in mouse liver after the intraperitoneal administration of D-glucuronolactone. 4. D-Glucarodilactone decomposed spontaneously in aqueous solution to give 25% of D-glucaro-(1→4)-lactone; the percentage conversion was unaffected by human-liver homogenates, but considerably increased by mouse-liver or pig-liver fractions.

The presence of D-glucaric acid in normal mammalian urine (Marsh, 1963a; Ishidate, Matsui & Okada, 1965; Aarts, 1965) has been ascribed to the action of a dehydrogenase, present in liver and other tissues, on D-glucuronolactone (Marsh, 1963b). It was suggested that the initial product of this enzymic oxidation was D-glucaro-(1→4)-(6→3)-dilactone, which is readily convertible into D-glucaro-(1→4)-lactone, a powerful β -glucuronidase inhibitor. The natural occurrence of the (1→4)-lactone, whether produced from endogenous free D-glucuronic acid or from administered D-glucuronolactone, would be of particular interest as an enzyme inhibitor of high specificity that could conceivably have a physiological role in the control of β -glucuronidase action.

In the present work, the products of action of liver D-glucuronolactone dehydrogenase from different species were examined under conditions that would preserve any D-glucaro-(1→4)-lactone from spontaneous hydrolysis. Since the instability of D-glucarodilactone (Smith, 1944) precluded the possibility of its isolation and identification, the inhibitory properties of the final reaction products were compared with those of the decomposition products of synthetic D-glucarodilactone.

A preliminary communication of part of this work has already been published (Marsh, 1965).

METHODS AND MATERIALS

Tissue sources and preparations. White mice were from Institute stock (Tyler's original, Mill Hill colony); the

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animals that were administered with D-glucuronolactone were all male, 10 weeks old, weight approx. 30g., and were starved for 24hr. before the treatment. Human-liver specimens, supplied by Dr A. J. Carr, Pathology Department, Aberdeen University, were from autopsy and were removed as soon as possible after death and stored at -20° . Fresh pig liver was obtained from Robert Lawson and Sons (Dyce) Ltd. Homogenates (10%, w/v) of these tissues were made in 0.17M-KCl and the particulate fraction was removed by centrifugation as described by Marsh & Carr (1965). Partially purified D-glucuronolactone dehydrogenase was made from the supernatants as described by Marsh (1963b). The standard rat-liver β -glucuronidase preparation (Marsh, 1963a) was prepared from liver tissue of adult rats (hooded Lister strain) and used in the enzyme assays at a dilution of 1.2–31./g. of moist liver, depending on the pH employed.

Materials. The sources of D-glucurono-(6→3)-lactone, potassium hydrogen D-glucarate, D-glucaro-(6→3)-lactone, D-glucaro-(1→4)-(6→3)-dilactone, NAD and phenolphthalein β -D-glucuronide were as previously described (Marsh, 1963c). D-Glucarate solution was obtained by titrating a solution of the potassium salt with alkali to pH 8.0. D-Glucaro-(1→4)-lactone monohydrate, m.p. 89–92°, was prepared by the method of Bose, Hullar, Lewis & Smith (1961).

Procedures. Measurement of total D-glucaric acid (i.e. acid plus lactones) was as described by Marsh (1963a), and of phenolphthalein liberated enzymically from its glucuronide as described by Levvy & Marsh (1959).

EXPERIMENTAL AND RESULTS

Spontaneous decomposition of D-glucaro-(1→4)-(6→3)-dilactone to a β -glucuronidase inhibitor. The inhibitory effect of D-glucarodilactone on β -glucuronidase was found to be variable, depending on the time between dissolution of the sample and its inclusion in the enzyme assay. This was due to

Fig. 1. Changes in the rates of hydrolysis of 0.63 mM-phenolphthalein β -glucuronide by the standard rat-liver preparation caused by the inclusion of 20 μ M-D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone. Incubations were at 38° in 0.05 M-phosphate buffer, pH 6.0 (O), 0.125 M-acetate buffer, pH 5.2 (Δ), or 0.125 M-acetate buffer, pH 4.5 ($\square$).

the formation of a strongly inhibitory entity, as demonstrated by a change in the degree of inhibition during the initial incubation period (Fig. 1). The dilactone was dissolved in the appropriate buffer at 0°, and substrate and enzyme preparation were added immediately afterwards. At pH 6.0 and 38°, inhibition by 20 μ M-D-glucarodilactone became maximal and constant after 30 min., whereas at lower pH values a constant inhibitory effect was attained after a somewhat longer period. Freshly prepared solutions of 20 μ M-D-glucarate or 20 μ M-D-glucaro-(6 $\rightarrow$ 3)-lactone had negligible inhibitory effects. It was therefore assumed that the inhibitor formed from D-glucarodilactone was D-glucaro-(1 $\rightarrow$ 4)-lactone, which has been shown (Smith, 1944) to be a product of decomposition in aqueous solution. It seemed doubtful whether the dilactone itself had any inhibitory effect at the concentration employed.

The maximum percentage inhibitions by D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone of the rate of hydrolysis of the substrate, shown in Fig. 1, were identical with those obtained when the dilactone was incubated for 1 hr. at 38° in buffer before the addition of substrate and enzyme, and then incubated for a further hour. From plots of the inhibition of rat-liver β -glucuronidase by different concentrations of D-glucaro-(1 $\rightarrow$ 4)-lactone, equivalent concentrations of this compound required to produce the observed maximum inhibitory effects of 20 μ M-D-glucarodilactone were derived, and were as follows: at pH 6.0, 5.2 μ M; at pH 5.2, 4.6 μ M; at pH 4.5, 5.0 μ M. Thus at these pH values approx. 25% of the dilactone was apparently converted into D-glucaro-(1 $\rightarrow$ 4)-lactone, which is stable under these conditions (Levvy, 1952).

Table 1. Effect of preincubation with liver-tissue fractions on the decomposition of D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone

The dilactone was incubated for 1 hr. at 38° in 0.05 M-phosphate buffer, pH 6.0, in the presence or absence of the soluble liver-homogenate fraction (final concn. 50 ml./g. moist weight), then phenolphthalein β -glucuronide (final concn. 0.63 mM) was added and the β -glucuronidase activity determined (dilactone final concn. 20 μ M). Inhibitions were determined by reference to similar assays lacking dilactone, and the equivalent D-glucaro-(1 $\rightarrow$ 4)-lactone contents were calculated from standard plots with the appropriate tissue fraction under similar conditions. Further details are given in the text.

Species	Presence or absence of enzyme	Inhibition of β -glucuronidase (%)	Equivalent concn. of (1 $\rightarrow$ 4)-lactone (μ M)
Man	+	47	6.4
Man	—	45	6.0
Mouse	+	71	13.2
Mouse	—	52	5.2
Pig	+	71	9.3
Pig	—	54	4.8

Effects of preincubation of D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone with liver-homogenate fractions. In the presence of the soluble fractions of iso-osmotic liver homogenates in 0.17 M-potassium chloride of some species, the production of β -glucuronidase inhibitor from the decomposition of D-glucaro-dilactone appeared to be considerably greater than normal. The dilactone was incubated for 1 hr. with the liver fractions at pH 6.0, then phenolphthalein β -glucuronide added and the apparent β -glucuronidase activity measured and compared with assays lacking the dilactone. The activity of this enzyme in pig liver was so low (see Conchie, Findlay & Levvy, 1959) that in this case it was necessary to add the standard rat-liver enzyme along with the substrate; the pig-liver extract was then also included in the controls. A pH value (6.0) higher than that for maximum enzyme activity (i.e. 4.5 or 5.2) was employed to prevent any conversion of D-glucarate into the inhibitory (1 $\rightarrow$ 4)-lactone during the incubation period.

The results of these experiments are shown in Table 1. After preincubation with the human-liver fraction, decomposition products of D-glucaro-dilactone had inhibitory activity identical with those from decomposition of the dilactone in buffer alone. However, in the presence of mouse-liver or pig-liver fractions the dilactone decomposed into products with considerably higher inhibitory power. There were differences in susceptibility to inhibition between the β -glucuronidases from different sources, and equivalent concentrations of D-glucaro-(1 $\rightarrow$ 4)-lactone necessary to give these

effects are also shown in Table 1. The formation of the (1→4)-lactone, if it is the inhibitory agent produced from the dilactone, was at least doubled in the presence of the mouse-liver or pig-liver fraction. Control experiments showed that the β -glucuronidase in the tissue fractions was not made more sensitive to inhibition by the (1→4)-lactone by preincubation with it in the absence of substrate. If the mouse-liver or pig-liver fractions were heated at 100° for 5 min. before incubation with the dilactone, potentiated inhibitory effects on β -glucuronidase were not observed; nor was any such effect apparent if bovine serum albumin, at similar protein concentration, was substituted for the liver fractions.

This potentiation of inhibition of the products from D-glucarodilactone therefore appears to be an enzymic effect, which is still apparent in dialysed mouse-liver or pig-liver fractions. No evidence could be obtained for the presence of a lactonase that produces the (1→4)-lactone from either D-glucaric acid or its (6→3)-lactone. Thus these two compounds were not inhibitors of β -glucuronidase after being incubated with the liver-homogenate fractions at pH 6.0.

Production of β -glucuronidase inhibitors by the action of D-glucuronolactone dehydrogenase. The influence of enzymic oxidation products of D-glucuronolactone on β -glucuronidase was studied with partially purified preparations of D-glucuronolactone dehydrogenase, from human and mouse liver, which also contained β -glucuronidase activity. The rate of hydrolysis of phenolphthalein β -D-glucuronide was almost unchanged over a period of 2 hr. at pH 6.0 and 38° when either D-glucuronolactone or NAD was included in the incubation mixture (Fig. 2); any production of the feebly

inhibitory D-glucuronic acid (Levy, 1952) by hydrolysis of its lactone thus had little effect. With the human enzyme, the rate of hydrolysis of phenolphthalein β -glucuronide decreased continuously when NAD and D-glucuronolactone were present together. The activity of D-glucuronolactone dehydrogenase in the liver preparation at this pH is considerable although not optimum (see the Discussion section) and the estimated total D-glucaric acid (free acid plus lactones) concentration was 60 μ M in the illustrated experiment after 2 hr., at which time the rate of hydrolysis was equivalent to inhibition by 16 μ M-D-glucaro-(1→4)-lactone. The addition to the incubation mixture of 10 mM-*n*-pentanol, a strong inhibitor of D-glucuronolactone dehydrogenase (Marsh, 1963c), caused this decrease in the rate of phenolphthalein β -glucuronide hydrolysis to disappear, and the total D-glucaric acid concentration was then 5 μ M after 2 hr.

A similar experiment with the mouse-liver preparation gave very different results. The rate of hydrolysis of the substrate over 3 hr. was then constant (as in the upper line of Fig. 2) and unchanged by the addition of D-glucuronolactone and NAD to the incubation mixture. There was no evidence therefore for the production of an entity inhibitory to β -glucuronidase in this case, although the total D-glucaric acid concentration produced after 3 hr. was found to be 53 μ M when the two substrates for D-glucuronolactone dehydrogenase were included.

The inhibitory properties of the diffusible products of the action of human-liver and mouse-liver D-glucuronolactone dehydrogenases were examined, and compared with those of the products from D-glucarate, D-glucaro-(1→4)-(6→3)-dilactone or D-glucaro-(6→3)-lactone incubated with the enzymes under the same conditions. The results are shown in Table 2. During the period of dialysis (48 hr. at 0°), any D-glucaro-(1→4)-(6→3)-dilactone formed during the initial incubation would be completely converted into the monolactones. The product from oxidation of D-glucuronolactone with the human-liver enzyme had a strong inhibitory effect, but this inhibition was not appreciably affected by the treatment with acid at 100°, which would convert any D-glucarate present partially into the (1→4)-lactone (Levy, 1952). However, inhibitory activity was destroyed by heating at a pH high enough to hydrolyse any lactones present, but was restored by subsequent acid treatment. This behaviour before and after the various treatments, was duplicated by the diffusible fraction from the incubation of D-glucaro-(1→4)-(6→3)-dilactone with the enzyme, whereas the products from incubations of D-glucarate or its (6→3)-lactone were almost non-inhibitory unless subjected to acid treatment, as was also the product

Fig. 2. Incubation of a human-liver preparation at pH 6.0 with 0.63 mM-phenolphthalein β -glucuronide (○), and in the presence of 5 mM-D-glucuronolactone (Δ), 2.5 mM-NAD (◼) or glucuronolactone + NAD (●).

Table 2. *Inhibitory effects of products of human-liver and mouse-liver D-glucuronolactone dehydrogenases*

Partially purified liver enzyme preparations (see the Methods and Materials section) were incubated with 5 mm-D-glucuronolactone and 2.5 mm-NAD, or with 0.25 mm-D-glucaric acid (or its lactones), in 0.05 M-phosphate buffer, pH 6.0 (total volume 10 ml.), for 2 hr. at 38°, then dialysed against 10 ml. of water for 48 hr. at 0°. In each case, the diffusate (2 ml.) was: (A) adjusted to pH 4.5; (B) adjusted to pH 4.0, heated for 40 min. at 100°, cooled and adjusted to pH 4.5; (C) adjusted to pH 7.5, heated for 15 min. at 100°, cooled and adjusted to pH 4.5; (D) adjusted to pH 7.5, heated for 15 min. at 100°, adjusted to pH 4.0, heated for 40 min. at 100°, cooled and adjusted to pH 4.5. Changes in pH were made with N-HCl or N-NaOH. The solutions (final volume 4.5 ml.) were tested as inhibitors (diluted 1:1) of β -glucuronidase at pH 5.2 with the standard rat-liver enzyme.

Incubated system	Inhibition of β -glucuronidase after treatment (%)			
	(A)	(B)	(C)	(D)
Human-liver prep. + D-glucuronolactone + NAD	59	57	6	53
Human-liver prep. + D-glucarate	7	71	8	70
Human-liver prep. + D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone	72	64	7	62
Human-liver prep. + D-glucaro-(6 $\rightarrow$ 3)-lactone	10	59	10	70
Mouse-liver prep. + D-glucuronolactone + NAD	6	54	7	72
Mouse-liver prep. + D-glucarate	8	61	9	62
Mouse-liver prep. + D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone	75	67	10	65
Mouse-liver prep. + D-glucaro-(6 $\rightarrow$ 3)-lactone	8	56	11	66

Fig. 3. β -Glucuronidase activities (○) and D-glucaric acid contents (●) of soluble mouse-liver-homogenate fraction after a single intraperitoneal injection of D-glucuronolactone (1 g./kg. body wt.). Enzyme assays were done at pH 6.0 in 0.05 M-phosphate buffer with 0.63 mm-phenolphthalein β -glucuronide. The concentration of enzyme preparation corresponded to 1.25% (w/v) moist tissue.

obtained with mouse-liver D-glucuronolactone dehydrogenase. Indeed, this last entity resembles more the product obtained from D-glucaro-(6 $\rightarrow$ 3)-lactone rather than that from D-glucarate, in that higher inhibitions were obtained after preliminary heating with alkali followed by acid treatment than after acid treatment alone.

Production of β -glucuronidase inhibitor in vivo by the administration of D-glucuronolactone. Single doses of D-glucuronolactone (1 g./kg. body wt.) were given intraperitoneally to mice, the animals were killed after intervals and the livers removed immediately. β -Glucuronidase activities of the

soluble fractions of liver homogenates in 0.17 M-potassium chloride were then measured at pH 6.0. A sharp fall in enzyme activity was observed 20 min. after treatment of the animals; after 40 min. enzyme activity commenced to rise again and within 2 hr. it had regained its original value (Fig. 3). That this change in the β -glucuronidase activity was due to the presence of an inhibitor, and not to a loss of the enzyme protein, was indicated by the fact that the relative magnitude of the change in enzyme activity varied with the concentration of the homogenate fraction in the assay. Whereas (total) D-glucaric acid was undetectable in normal livers, or in the livers of mice immediately after the injection of D-glucuronolactone, it was subsequently present in the tissue for periods up to 4 hr. after the treatment (Fig. 3). Maximum liver concentrations of D-glucaric acid were observed when the β -glucuronidase activity was minimal, suggesting that the factor inhibiting β -glucuronidase that developed after the treatment was D-glucaro-(1 $\rightarrow$ 4)-lactone.

DISCUSSION

The chemical formulae and transformations of compounds that may be implicated in this work are shown in Scheme 1. The D-glucaro-(1 $\rightarrow$ 4)-(6 $\rightarrow$ 3)-dilactone (IV) that was used was a non-crystalline syrup obtained by dehydration of D-glucaro-(1 $\rightarrow$ 4)-lactone (III) (Smith, 1944). Harigaya (1964a) and Hirasaka & Umemoto (1965) have reported the isolation of crystalline products by this reaction and by dehydration of D-glucaro-(6 $\rightarrow$ 3)-lactone (V)

Scheme 1. Chemical (→) and enzyme-mediated (---→) reactions of D-glucuronolactone and D-glucaric acid derivatives.

with identical properties, and the structure was confirmed as the dilactone (IV) by the infrared spectrum and by the susceptibility of the acetylated derivative to periodic acid oxidation after delactonization. Smith (1944), however, obtained a solid product from the (6→3)-lactone (V) that he assumed to contain a (1→5)-lactone ring and that he formulated as the dilactone (VI); his product inhibited β -glucuronidase *in vitro* only to the same degree as the (6→3)-lactone (V) (Levy, 1952), to which reconversion in aqueous solution might be expected owing to the greater lability of the δ -lactone ring. The two five-membered rings of the dilactone (IV) should not differ greatly in stability, and on partial hydrolysis the compound (IV) would be expected to yield both the (1→4)-lactone (III) and the (6→3)-lactone (V). By determination of the resulting lactones, which they separated by paper chromatography, Hirasaka, Umemoto,

Sukegawa & Matsunaga (1965) found that the dilactone (IV) produced about 30% of the (1→4)-lactone (III) and 60% of the (6→3)-lactone (V) when kept in aqueous solution at room temperature for 5 hr. These results agree fairly well with the spontaneous maximum conversion of the dilactone (IV) into 25% of the (1→4)-lactone (III) in about 1 hr. at 38°, calculated by the development of inhibitory power. By enzymic analysis similar to that described here, Harigaya (1964a) also concluded that the inhibitory power of the dilactone (IV) was due to its decomposition to the (1→4)-lactone (III), although traces of the dilactone (IV) were still recognizable chromatographically even after 24 hr. standing in aqueous solution. However, the dilactone (IV) is apparently stable only in equilibrium with D-glucaric acid (VII) and its monolactones at temperatures above 90° (Hirasaka *et al.* 1965).

The results obtained with the human-liver preparation were consistent with the hypothesis (Marsh, 1963b) that D-glucuronolactone dehydrogenase acts on D-glucofuranurono-(6→3)-lactone (I) to produce the dilactone (IV), which subsequently decomposes spontaneously in aqueous solution to the monolactones (III) and (V). Harigaya (1964b) found that the dilactone (IV) produces more powerful inhibition than the (1→4)-lactone (III) of liver β -glucuronidase after its oral or intravenous administration to mice, presumably because it lacks a strongly ionizable group and can therefore easily penetrate the intact cell before decomposition to the (1→4)-lactone (III). The production of the (1→4)-lactone (III) *in vivo* after the oral administration of D-glucuronolactone to man would also appear to be the only satisfactory explanation for the rapid fall in blood β -glucuronidase activity obtained by Fishman *et al.* (1951).

Experiments with mouse liver yielded results qualitatively different from those with human liver and present anomalies that are not readily explicable. According to Harigaya (1964b) the oral administration of D-glucuronolactone to mice causes little change in liver β -glucuronidase activity, whereas I find that intraperitoneal administration causes rapid formation of a strong β -glucuronidase inhibitor, presumably the (1→4)-lactone (III). In contrast, experiments *in vitro* suggest that the stable product of mouse-liver D-glucuronolactone-dehydrogenase action is either the (6→3)-lactone (V) or glucaric acid (VII), and no evidence of the presence of a lactonase was obtainable to account for the apparent absence of the (1→4)-lactone (III), although the existence of a lactonizing enzyme able to accomplish the formation of the (1→4)-lactone (III) from glucaric acid (VII) is suggested by the inhibition of mouse-liver β -glucuronidase that Harigaya (1964b) observed after the oral administration of the (6→3)-lactone (V). A possibility suggested by Professor M. Ishidate (personal communication) is that mouse-liver dehydrogenase acts not on the furanose form (I) but on the aldehydic form (II) of D-glucuronolactone to give the (6→3)-lactone (V). The dehydrogenase preparations from mouse liver and human liver were both found to have optimum activities at pH 7.0–7.3, this value being somewhat higher than that previously found for the partially purified enzyme from rat liver, at pH 6.5–6.6 (Marsh, 1963c).

The apparent ability of mouse-liver and pig-liver

preparations to catalyse the production of the (1→4)-lactone (III) from the dilactone (IV) is another point of difference from the human-liver preparations. The effect could be interpreted either as activation of the hydrolysis of the (6→3)-lactone ring of the dilactone (IV) or as suppression of hydrolysis of its (1→4)-lactone ring, without, however, affecting the activity of the β -glucuronidase inhibitor produced. The lability of the dilactone (IV), due to the presence of two lactone groups, may result in its reaction with free amino groups of specific proteins to produce unstable amide linkages. The factor causing these potentiating effects may be so labile as to be absent from the human-liver preparations, which were inevitably prepared from material not so fresh as the other animal tissues. The presence of unstable lactonases, not identifiable by the techniques used here, might also explain the apparent formation *in vivo* of D-glucaro-(1→4)-lactone, in contrast with its demonstrated absence from the reaction products of mouse-liver D-glucuronolactone dehydrogenase.

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REFERENCES

- Aarts, E. M. (1965). *Biochem. Pharmacol.* **14**, 359.
Bose, R. J., Hullar, T. L., Lewis, B. A. & Smith, F. (1961). *J. org. Chem.* **26**, 1300.
Conchie, J., Findlay, J. & Levvy, G. A. (1959). *Nature, Lond.*, **183**, 615.
Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C. & Homburger, F. (1951). *J. clin. Invest.* **30**, 685.
Harigaya, S. (1964a). *J. Biochem., Tokyo*, **56**, 392.
Harigaya, S. (1964b). *J. Biochem., Tokyo*, **56**, 400.
Hirasaka, Y. & Umamoto, K. (1965). *Chem. pharm. Bull., Tokyo*, **13**, 325.
Hirasaka, Y., Umamoto, K., Sukegawa, M. & Matsunaga, I. (1965). *Chem. pharm. Bull., Tokyo*, **13**, 677.
Ishidate, M., Matsui, M. & Okada, M. (1965). *Analyt. Biochem.* **11**, 176.
Levy, G. A. (1952). *Biochem. J.* **52**, 464.
Levy, G. A. & Marsh, C. A. (1959). *Advanc. Carbohydr. Chem.* **14**, 381.
Marsh, C. A. (1963a). *Biochem. J.* **86**, 77.
Marsh, C. A. (1963b). *Biochem. J.* **87**, 82.
Marsh, C. A. (1963c). *Biochem. J.* **89**, 108.
Marsh, C. A. (1965). *Abstr. 2nd Meet. Fed. Europ. biochem. Soc., Vienna*, p. 23.
Marsh, C. A. & Carr, A. J. (1965). *Clin. Sci.* **28**, 209.
Smith, F. (1944). *J. chem. Soc. p.* 633.