Studies in Detoxication

67. THE BIOSYNTHESIS OF THE GLUCURONIDES OF UMBELLIFERONE AND 4-METHYLUMBELLIFERONE AND THEIR USE IN FLUORIMETRIC DETERMINATION OF β-GLUCURONIDASE*

By J. A. R. MEAD, J. N. SMITH and R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(*Received* 2 *June* 1955)

Umbelliferone (7-hydroxycoumarin) and its conjugates appear to be metabolites of coumarin in the rabbit (Mead, Smith & Williams, 1955). It was observed that, although umbelliferone fluoresced strongly in ultraviolet light at pH 9–10, its conjugates showed little or no fluorescence. The glucuronide and ethereal sulphate of umbelliferone were therefore made and the former was found to be practically non-fluorescent. The possibility that these conjugates could be used as substrates for the determination of β-glucuronidase and arylsulphatase was therefore investigated. Since umbelliferone is highly fluorescent it appeared likely that these conjugates could be used for the determination of the enzymes in very small amounts of material. The present communication deals with the use of the glucuronides of umbelliferone and the cheaper 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) for the determination of β-glucuronidase. (This work has been briefly reported by Mead, Smith & Williams, 1954.) The ethereal sulphates of umbelliferone and 4-methylumbelliferone show a fluorescence which is considerably less than the corresponding hydroxycoumarins. We have attempted to use these compounds for the fluorimetric assay of arylsulphatase, but we do not consider them satisfactory because their fluorescence is sufficient to give relatively high blank values. The β-glucosides of umbelliferone and 4-methylumbelliferone, however, have been found to be satisfactory for the determination of β-glucosidase, and an account of these compounds will be given later.

EXPERIMENTAL

Materials. Umbelliferone, m.p. 224–226°, purchased from British Drug Houses Ltd., had a pale-reddish colour, and paper chromatography showed that it was not pure. In *n*-butanol–acetic acid–water (4:1:5 by vol.), besides a main fluorescent spot due to umbelliferone, three other much weaker fluorescent spots were present. As a standard for fluorescent purposes umbelliferone was purified by way of the sulphuric ester. The umbelliferone (4·5 g.) was dissolved in pyridine (10 ml.) and a solution of chlorosulphonic acid (3 g.) in pyridine (10 ml.) added carefully with cooling. The mixture was then kept at room temp. for 24 hr. Water (100 ml.) was then added, followed by a slight excess of $KHCO_3$. The precipitate which formed was collected by filtration, dissolved in the minimum of hot water (charcoal) and the solution filtered. On cooling the *potassium umbelliferone sulphate* (*potassium* 2-*oxo*-1:2-*benzopyran*-7-*yl sulphate*) separated and was recrystallized from 80% (v/v) aqueous ethanol as colourless needles (yield 2 g.). (Found: K, 14·0; S, 11·3. $C_9H_5O_6SK$ requires K, 13·9; S, 11·4%.) Short hydrolysis (10 min.) of this salt with 0·3 N-HCl and cooling yielded pure umbelliferone as colourless needles, m.p. 230–231°.

4-Methylumbelliferone, m.p. 181–183° (British Drug Houses Ltd.) was yellowish in colour. Paper chromatography showed it to contain no fluorescent impurities. It was nevertheless purified as for umbelliferone. *Potassium 4-methylumbelliferone sulphate* (*potassium* 4-*methyl*-2-*oxo*-1:2-*benzopyran*-7-*yl sulphate*) was prepared from 4-methylumbelliferone (4·5 g.) as described above for the umbelliferone derivative. It formed colourless needles from 80% ethanol (yield 2 g.). (Found: K, 13·3; S, 10·7. $C_{10}H_7O_6SK$ requires K, 13·1; S, 10·9%.) Hydrolysis of the salt yielded pure 4-methylumbelliferone as colourless needles, m.p. 185–186°.

Umbelliferone glucuronide. In two quantitative experiments it was found that 42 and 39% respectively of an oral dose of 0·2 g. of umbelliferone/kg. was excreted as conjugated glucuronic acid, and 18·6 and 21·6% respectively as conjugated sulphate. Six rabbits were each given by stomach tube 2 g. of commercial umbelliferone suspended in water. The urine (750 ml.) collected during the next 24 hr. was almost neutral (pH 7–7·5), was non-reducing and gave a strong naphthoresorcinol reaction. The glucuronide fraction of the urine was separated by systematic lead acetate precipitation (cf. Kamil, Smith & Williams, 1951) and the filtrate, obtained after removal of lead with H_2S at the basic lead acetate stage, was evaporated to 150 ml. under diminished pressure at 40°. On keeping the solution overnight at 0°, a crystalline precipitate separated. This was collected and recrystallized from 70 ml. of hot water (charcoal) to yield 0·91 g. (3·1% of the dose) of *umbelliferone glucuronide* (2-*oxo*-1:2-*benzopyran*-7-*yl* β-D-*glucosiduronic acid*, or 7-β-D-*glucuronosidocoumarin*). It was finally purified from absolute ethanol, from which it crystallized as colourless needles of the monohydrate, m.p. 149–150° and $[\alpha]_D^{20}$ −105° in water (*c*, 1). (Found: C, 50·5; H, 4·7; loss at 110°, 5·1. $C_{15}H_{14}O_9,H_2O$ requires C, 50·6;

* Part 66: Jondorf, Parke & Williams (1955).

H, 4·5; H_2O, 5·1%.) *Methyl* (*2-oxo*-1:2-*benzopyran*-7-*yl tri*-O-*acetyl*-β-D-*glucosid*)*uronate*, prepared in the usual way, was obtained as a white microcrystalline powder from light petroleum (b.p. 120°), m.p. 181–182° and $[\alpha]_D^{20}$ −44° in $CHCl_3$ (*c*, 1). (Found: C, 55·5; H, 4·6. $C_{22}H_{22}O_{12}$ requires C, 55·2; H, 4·6%.) The yield of crystalline glucuronide was low in most experiments and much non-crystalline glucuronide gum was obtained from which the triacetyl methyl ester could be isolated.

Hydrolysis of the glucuronide with 2N-HCl or with locust crop liquor containing β-glucuronidase yielded umbelliferone. The umbelliferone was detected chromatographically on paper by its fluorescence. A spot of the hydrolysate was chromatographed on Whatman no. 1 paper, using *n*-butanol–benzene–ammonia, sp.gr. 0·88 (2:5:2 by vol.). In this solvent umbelliferone has R_F 0·48 and the glucuronide R_F 0·02. The umbelliferone spot was readily detected by its bright-blue fluorescence in ultraviolet light after the paper had been subjected to ammonia fumes.

4-Methylumbelliferone glucuronide. In three quantitative experiments it was found that 45, 51 and 52% respectively of an oral dose (0·2 g./kg.) of 4-methylumbelliferone was excreted as conjugated glucuronic acid, and 4, 6 and 7% respectively as conjugated sulphate. Two rabbits were each given 2·5 g. of commercial 4-methylumbelliferone as described for umbelliferone. The non-reducing, slightly alkaline 24 hr. urine (300 ml.) was worked up by the lead acetate procedure to the stage of removal of lead from the basic lead acetate precipitate with H_2S. The filtrate (400–500 ml.) from lead sulphide was kept at 0° overnight and hydrated *4-methylumbelliferone glucuronide* (*4-methyl-2-oxo*-1:2-*benzopyran*-7-*yl* β-D-*glucosiduronic acid*) separated (2·3 g.). Concentration of the mother liquors to small bulk *in vacuo* yielded a further 0·24 g. (yield 20–25% of dose). The compound was twice recrystallized from water and formed hydrated colourless needles, m.p. 139–141° (decomp.) after sintering at 100–105° and $[\alpha]_D^{23}$ −105° in water (*c*, 0·25). Elementary analysis of three different air-dried preparations were consistent. (Found: C, 46·3, 46·6, 46·8; H, 5·7, 5·9, 5·3. $C_{16}H_{16}O_9,3{\cdot}5H_2O$ requires C, 46·3; H, 5·6%.) On heating at 110°, even for prolonged periods, the compound could not be completely dehydrated. (Found: loss at 110°, 13·8, 14·2, 14·1. $3{\cdot}5H_2O$ requires 15·2 and $3H_2O$ 13%.) The dehydrated substance regained some water and its composition was approximately that of a hemihydrate, m.p. 139–140° (decomp.). (Found: C, 52·6; H, 5·0. $C_{16}H_{16}O_9,0{\cdot}5H_2O$ requires C, 53·2; H, 4·7%.) On heating the glucuronide for 30 min. in 2N-HCl, and then cooling, 4-methylumbelliferone (m.p. and mixed m.p. 186°) crystallized from the hydrolysate. *Methyl* (*4-methyl-2-oxo*-1:2-*benzopyran*-7-*yl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* was prepared in the usual manner and formed white needles from absolute ethanol, m.p. 189–190° and $[\alpha]_D^{20}$ −45° in $CHCl_3$ (*c*, 1). (Found: C, 56·1; H, 4·9. $C_{23}H_{24}O_{12}$ requires C, 56·1; H, 4·9%.)

The determination of β-glucuronidase

An H553 Spekker fluorimeter with 20 ml. cuvettes was used. The secondary filters were made of Chance O.B. 1 blue glass.

pH–*fluorescence curves.* From the curves published by Goodwin & Kavanagh (1950, 1952) for umbelliferone and 4-methylumbelliferone it is not possible to determine the precise pH at which maximum fluorescence is developed. The curves were therefore redetermined in buffers of pH range 5·8–11·0, with aqueous solutions containing 0·04–0·05 μg./ml. of umbelliferone or 4-methylumbelliferone, fluorescence being measured against standard solutions of quinine bisulphate (2 or 4 μg./ml.) in 0·1N-H_2SO_4. The curves show that the maximum fluorescence of umbelliferone is reached at about pH 9·5 and of 4-methylumbelliferone at about pH 10. There is no change in the intensity on raising the pH to 11. At about pH 10 the fluorescence is constant for at least 1 hr. At pH 11·76 (glycine buffer) there was a rapid drop in the intensity of fluorescence, presumably owing to hydrolysis of the coumarins. It was decided finally to use a glycine buffer of pH 10·32 as the medium for developing fluorescence. In this buffer the fluorescence of 4-methylumbelliferone was stable for at least 12 hr. if not irradiated for long periods with ultraviolet light.

Preparation of the standard curve. The fluorescence intensities of umbelliferone and 4-methylumbelliferone are roughly 20 times that of quinine bisulphate. When the instructions of Bowen & Wokes (1953) for the determination of density difference curves were followed it was found that the concentration ranges of the hydroxycoumarins over which linearity of response could be expected with our instrument were about 0·01–0·10 μg./ml. Empirically it was found that the quinine hydrogen sulphate concentration that would give drum readings of 0·1–0·8 against these concentrations of the hydroxycoumarins was 4 μg./ml.

Aqueous solutions of the hydroxycoumarins containing 5 μg./ml. were prepared. Known volumes were diluted to 50 ml. with 0·1M glycine buffer (pH 10·32) and the fluorescence intensities measured against a quinine bisulphate standard containing 4 μg./ml. in 0·1N-H_2SO_4. The drum readings of the fluorimeter were plotted against the concentration of the hydroxycoumarin, on logarithmic paper. A straight line was obtained.

Recovery of 4-methylumbelliferone. The hydroxycoumarin added to tissue homogenates could be recovered almost quantitatively by measuring the fluorescence in the glycine buffer. Some of the recovery experiments are quoted in Table 1.

Enzyme solutions. Animal tissues (5–100 mg. according to the expected activity) were homogenized with 1 ml. of ice-water in a Potter–Elvehjem glass homogenizer, or ground with sand in a mortar with 2–3 ml. of ice-water and the product diluted to 100 ml. (10 ml. with tissues of low activity) with distilled water. Locust crop liquor (Robinson, Smith & Williams, 1953) was usually diluted 25000 times with water. Urine (1 ml.) was used as such, and saliva diluted about four times with water.

Substrate solution. For most experiments the stock substrate consisted of a 0·001M aqueous solution of hydrated 4-methylumbelliferone glucoronide. (In the enzyme assays the final substrate concentration was 0·0001M.) This solution was stable for 6 weeks if kept at 0°, but it deteriorated in 2–3 days at room temperature. A few experiments were carried out with umbelliferone glucuronide in similar concentration.

Buffer and other solutions. Most experiments were carried out with 0·1M acetate buffers but 0·1M phosphate–citrate and Michaelis's veronal–acetate–HCl buffers were used in some experiments. For the measurement of fluorescence, a 0·1M glycine buffer of pH 10·32, a solution of quinine bisulphate (4 μg./ml.) in 0·1N-H_2SO_4 and a 1 μg./ml. aqueous solution of recrystallized 4-methylumbelliferone were prepared.

Table 1. *Fluorimetric recovery of 4-methylumbelliferone from tissues*

For liver 30 mg. was homogenized with 1 ml. of water and then diluted to 100 ml. with water. A portion (1 ml.) of this solution was mixed with 3·5 ml. of acetate buffer (pH 4·6) and 0·5 ml. of aqueous 4-methylumbelliferone solution of the appropriate concentration, and made up to 25 ml. with glycine buffer, pH 10·32.

For muscle 90 mg. was homogenized with water and then diluted to 10 ml. with water. A portion (1 ml.) of this solution was then treated as for the liver homogenate.

Tissue	4-Methylumbelliferone Added (μg./ml.)	4-Methylumbelliferone Recovered (μg./ml.)
Guinea-pig liver		
Without incubation	0·02	0·02, 0·02, 0·02
	0·06	0·06, 0·06, 0·061
Incubated for 30 min. at 37°	0·02	0·019, 0·0195, 0·0195
	0·06	0·059, 0·060, 0·061
Guinea-pig skeletal muscle		
Without incubation	0·02	0·02, 0·02, 0·02
	0·06	0·057, 0·062, 0·061
Incubated for 30 min. at 37°	0·02	0·0195, 0·02, 0·02
	0·06	0·06, 0·062, 0·061

Assay of β-glucuronidase. Most of the measurements were carried out at pH 4·6 in acetate buffer. The enzyme solution (1 ml.) was mixed in a test tube with 3·5 ml. of 0·1 M acetate buffer of the required pH at 37° and 0·5 ml. of the 0·001 M substrate solution. The tubes were incubated usually for 0·5 hr. at 37°. The time of incubation should be measured accurately owing to the sensitivity of the method. Glycine buffer (20 ml.) was then added to each tube and the contents were mixed. The fluorescence of the solution was then determined against the quinine bisulphate standard. The blank tubes set up consisted of substrate solution (0·5 ml.) and acetate buffer (3·5 ml.). After incubation the blank was diluted with 19 ml. of glycine buffer and then 1 ml. of the enzyme solution and 1 ml. of the 4-methylumbelliferone solution were added. After mixing, the fluorescence of the blank solution was measured as before. The addition of 4-methylumbelliferone to the blank test was necessary in order to bring the value of the blank fluorescence on the calibration curve. The values of the blank were usually of the order of 0·001–0·003 μg. of 4-methylumbelliferone/ml., and were significant only when small amounts (e.g. 0·01 μg./ml.) of the methylumbelliferone were being measured.

β-Glucuronidase in blood. Fishman, Springer & Brunetti (1948) have reported on the occurrence of β-glucuronidase in blood, where it appears to be concentrated in the white cells. Attempts were therefore made to apply the fluorimetric method to blood. Owing to quenching of fluorescence by haemoglobin, the estimation of the enzyme in whole blood was not possible. Similar difficulties arose with highly coloured urines and plasma in which slight haemolysis had occurred, except in the case of plasmas of relatively high glucuronidase content (e.g. rabbit plasma) where the quenching substances could be eliminated by dilution. The glucuronidase activity of haemoglobin-free plasma could be readily assayed fluorimetrically. The assay was carried out essentially as described in the preceding paragraph, using a veronal–acetate–HCl buffer. Precipitation of plasma proteins occurred if phthalate or phosphate buffers were used. Human plasma was diluted 1 in 10 with distilled water, rat and guinea-pig plasma 1 in 25 and rabbit plasma 1 in 50, and the incubation time was extended to 1·5–3 hr.

RESULTS

Crude locust crop liquor with umbelliferone glucuronide as substrate showed an optimum pH at about 4·5–4·6 in acetate buffer and at 5·2–5·3 in citrate–phosphate buffer. When 4-methylumbelliferone glucuronide was used there was a broad optimum between 4·5 and 5·5 in acetate buffer and between 5·0 and 5·5 in citrate–phosphate buffer. The optimum substrate concentrations for the crop liquor in acetate buffer at pH 4·6 was found to be 0·0001 M, with little change up to 0·0002 M for umbelliferone glucuronide, and about 0·0001–0·0002 M for 4-methylumbelliferone glucuronide. Under standard conditions the amount of 4-methylumbelliferone liberated from the glucuronide by a constant amount of a given specimen of locust crop liquor was proportional to the time of incubation up to 90 min., the limit of time studied. Furthermore, with varying dilutions of crop liquor the liberation of 4-methylumbelliferone at pH 4·6 in 0·5 hr. was inversely proportional to the dilution of the enzyme solution. β-Glucuronidase is strongly inhibited by D-saccharo-1:4-lactone and by solutions of potassium hydrogen saccharate which have been boiled for 30 min. at pH 3·5, the inhibition by the saccharate solution being due to the formation of the lactone (Levvy, 1952). The glucuronidase of locust crop liquor acting on 4-methylumbelliferone glucuronide at pH 4·6 was also inhibited by boiled saccharate solutions, 50% inhibition occurring with 0·00002 M saccharate solutions and complete inhibition at 0·001–0·005 M. Heparin at a concentration of 0·4 mg./ml. had no inhibitory effect on the locust enzyme.

Crude homogenates of rat and guinea-pig liver in acetate buffer and with 4-methylumbelliferone glucuronide as substrate showed an optimum pH at

Table 2. *β-Glucuronidase activity of various tissues determined fluorimetrically*

S indicates tissue ground with sand and made up to 100 ml. with water. All the tissues were from the same animal of the species mentioned. G indicates tissue homogenized in a glass homogenizer and made up to 100 ml. with water. All tissues (excluding the foetal and young liver) were from the same animal but different from the one under S. Figures in parentheses indicate the volume to which the homogenized tissue was diluted if not 100 ml.

Tissue	Wt. of tissue taken (S) (mg.)	4-Methylumbelliferone liberated at pH 4·6 (μg./mg./hr.)	Wt. of tissue taken (G) (mg.)	4-Methylumbelliferone liberated (μg./mg./hr.) at pH 4·6	3·94
Guinea pig					
Liver	11·6	4·3	30	8·6	—
Foetal liver	—	—	28	9·8	—
Spleen	7·4	3·7	30	6·9	—
Adrenal	52·4	0·3	—	—	—
Skeletal muscle	445·3	0·04	87 (11)	0·06	—
Rat					
Liver, adult	30	12·9	13·2	22·5	25·4
Liver, young	—	—	22·5	14·7	—
Spleen	31·8	11·8	17·4	14·5	20·5
Testis	863·2	0·1	—	—	—
Skeletal muscle	827·4	0·07	100 (10)	0·19	0·24
Phrenic nerve	12·2 (10)	0·5	—	—	—
Housefly					
Whole flies	36·0 (11)	2·1	—	—	—
	34·5 (10)	2·8	—	—	—
	34·1 (25)	1·8	—	—	—

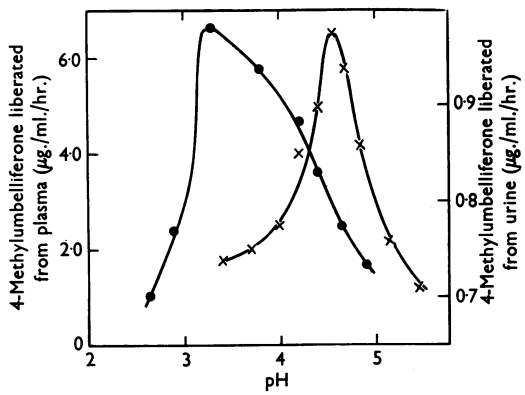

Fig. 1. pH–activity curves for the β-glucuronidase activity of human plasma and urine determined fluorimetrically with 4-methylumbelliferone glucuronide as substrate. ●, Fresh human plasma diluted ten times with water; measurements in veronal–acetate–HCl buffers. ×, Fresh human urine, undiluted in acetate buffer.

about pH 4. The pH–activity curve for the β-glucuronidase activity of a sample of human urine, where the optimum is at about pH 4·6 in acetate buffer, is shown in Fig. 1, which also shows the pH curve for human plasma in veronal–acetate–HCl buffer. Rabbit plasma appeared to be many times more active than human, guinea-pig or rat plasma.

Some of the results obtained by the fluorimetric method on various animal tissues are shown in Table 2. Our earlier measurements were carried out at pH 4·6 in acetate buffer with tissues which had been ground with sand. Higher values were obtained with tissues homogenized in a glass homogenizer. Some values are quoted at pH 3·94, which was near the optimum pH of the enzyme with 4-methylumbelliferone glucuronide as substrate. In Table 3 results are given for fresh human urine. The values are very small (1·0–1·5 μg./ml./hr.) and optimum activity was at about pH 4·6 in acetate buffer. Some results for plasma are given in Table 4. Optimum activity in guinea-pig, rat

Table 3. *β-Glucuronidase activity of human male urine*

Urine (1 ml. fresh random morning samples) used as described in text; 2·5 ml. of saliva diluted to 10 ml. and 1 ml. of solution used.

Subject	4-Methylumbelliferone liberated at pH 4·6 in acetate buffer (μg./ml./hr.)
Urine	
1	1·58, 1·80, 0·90*
2	1·50
3	1·20
4	1·80
5	0·50
6	1·15
7	1·15, 1·05*
8 (infant)	0·78
9 (female)	1·17, 1·40*
Saliva	
1	0·8, 0·45*

* Different samples.

Table 4. *β-Glucuronidase activity in plasma*

Measurements in acetate buffer unless stated otherwise. Figures in parentheses indicate volume to which plasma was diluted.

Species	Vol. of plasma taken and dilution (ml.)	4-Methylumbelliferone liberated (μg./ml./hr.) At optimum pH		At pH 4·6
Human	1	13	pH 3·4	5·6
	1	11		—
	1* (10)	7		2·5
Guinea-pig	1* (25)	8·7	pH 4·0	5·6
Rat	1* (25)	7·1	pH 4·4	6·4
Rabbit	1 (25)	137	pH 4·0	91 (fresh)
	1 (25)	145		92 (kept 24 hr. at 0°)
	1 (100)	140		—
	1 (100) arterial	115	pH 4·0	—
	1 (100) venous	115		—
	1* (50)	50		34

* Veronal–acetate–HCl buffer.

and rabbit plasma was found at about pH 4·0, but in four human plasmas the optimum was found at about pH 3·3–3·4. Measurements at pH 4·6 are also quoted for comparative purposes (cf. Fishman *et al.* 1948). Rabbit plasma was considerably more active than the plasmas of the other species examined, and there appeared to be no difference between arterial and venous blood. At pH 7·4 the enzyme was almost inactive.

DISCUSSION

Most of the results with animal tissues were obtained at pH 4·6, which is not the optimum value for the enzyme activity in liver, with 4-methylumbelliferone glucuronide as substrate. At pH 4 the liver enzyme would be expected to be nearly twice as active as at pH 4·6. The values for tissues homogenized in the glass homogenizer are greater than those obtained by grinding with sand, probably owing to better solution of the enzyme (cf. Walker & Levvy, 1953). However, the object of this paper was to demonstrate the use of a fluorigenic substrate for the assay of β-glucuronidase. It is claimed that the method is rapid and can be used for small amounts of tissue or for material with only weak glucuronidase activity, since 4-methylumbelliferone can be accurately determined fluorimetrically in minute amounts. Thus the glucuronidase content of small pieces of nerve or that of 1 ml. of normal urine, which has a low glucuronidase content, could be readily estimated with only 0·5 hr. incubation time. With some samples of locust crop liquor, 100 000- to 200 000-fold dilution was necessary before their glucuronidase content could be measured conveniently. Furthermore, the substrate 4-methylumbelliferone glucuronide can be isolated without difficulty from urine, since it crystallizes readily from the filtrate obtained after removal of lead from the basic lead acetate precipitate of the urine of rabbits receiving 4-methylumbelliferone.

SUMMARY

1. The glucuronides of umbelliferone and 4-methylumbelliferone have been prepared by feeding these hydroxycoumarins to rabbits. The triacetyl methyl esters of the glucuronides have also been described. The potassium salts of umbelliferone and 4-methylumbelliferone sulphuric esters have also been synthesized.

2. The glucuronides of umbelliferone and 4-methylumbelliferone are practically non-fluorescent, whereas the corresponding hydroxycoumarins are very highly fluorescent at pH 10–11, and can be measured fluorimetrically in quantities of 0·01 μg./ml. or less.

3. 4-Methylumbelliferone glucuronide is easily prepared and can be used as a substrate for β-glucuronidase. A fluorimetric method is described for the assay of β-glucuronidase. The method is highly sensitive and is applicable to very small amounts of tissue or materials of low glucuronidase content.

The expenses of this work were in part defrayed by a grant from the Medical Research Council, and one of us (J.A.R.M.) is grateful to the Council for a maintenance grant.

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Oxidative Metabolism in *Ascaris lumbricoides* from the Pig

By L. RATHBONE*
Department of Biochemistry, University College, London

(*Received* 23 *March* 1955)

The oxygen tension of the pig intestine is thought to be low (Bueding, 1949), and for this reason it has often been doubted whether oxygen is essential to *Ascaris lumbricoides*. Many workers have demonstrated an uptake of oxygen by *Ascaris* and rates up to 500 μl./g. (wet wt.) of whole worm per hr. have been observed in an atmosphere of oxygen (Grembergen, Damme & Vercruysse, 1949). Laser (1944) has shown that prolonged anaerobiosis causes an oxygen debt. Grembergen *et al.* (1949) and Bueding & Charms (1952) have demonstrated a succinoxidase system in preparations of muscle from *A. lumbricoides*, and Bueding & Charms report that this is of an unusual kind, since experiments failed to reveal the existence of a cytochrome system in the tissues.

EXPERIMENTAL

Tissue preparations. Worms were obtained from the slaughter-house. The specimens, after having been washed in warm water, were conveyed to the laboratory in large vacuum flasks containing 'keeping medium' (Baldwin & Moyle, 1947) at 38°. The worms were transferred to fresh medium on arrival at the laboratory and again after 24 hr. They were used on the day of removal from the host or on the succeeding day, except in cases in which it had been shown that activity in preparations from third-day worms was similar to that in first-day preparations.

Whole suspensions of muscle in 0·5% KCl solution were prepared with an homogenizer (Potter & Elvehjem, 1936). A portion (1 ml.) containing 0·4–0·6 g. (wet wt.) of tissue was added to each flask.

A particulate fraction was isolated from muscle by a method based on that of Schneider (1948), 0·2M sucrose being used. A 20% (wet wt./vol.) suspension of muscle in 0·2M sucrose was prepared and centrifuged at 600 *g* for 10 min. at 2°. The supernatant was decanted and centrifuged at 18000 *g* for 10 min. in the cold. The residue from about 60 ml. of original suspension was triturated with about 15 ml. of 0·2M sucrose solution. Such a suspension contained about 0·5–1·0 mg. of N/ml.

Acid-precipitated suspensions were prepared as follows. A 20% (wet wt./vol.) suspension of muscle in 0·05M phosphate buffer at pH 7·4 was centrifuged at 600 *g* for 10 min. at 2°. The supernatant was adjusted to pH 5·7 with N acetic acid and the precipitate collected immediately by centrifuging at 1000 *g* for 20 min. at 2°. The precipitate was suspended in an equal volume of 0·02M phosphate buffer at pH 7·4.

Materials. Succinic acid was recrystallized three times from hot water. The succinic acid was dissolved and neutralized, and the sodium salt precipitated with ethanol and recrystallized from 80% ethanol. Citric acid, α-oxoglutaric acid and L-malic acid were commercial products (L. Light and Co. Ltd.). Sodium fumarate and *cis*-aconitic acid were gifts from Dr K. R. Rees. The acids were neutralized with NaOH before use. Diphosphopyridine nucleotide (DPN) was prepared by a method described by LePage (1949). Adenosine triphosphate (ATP) was prepared by the method of Dounce *et al.* (1948). Cytochrome *c* was prepared from beef hearts by the method of Keilin & Hartree (1937). Hexokinase was prepared by the method of Berger, Slein, Colowick & Cori (1946) carried to the second alcohol-precipitation stage. The enzyme was dissolved in 1% glucose and stored at −10°. Catalase prepared from blood by the method of Herbert & Pinsent (1948) was a gift from Miss L. Hopkinson. It was assayed by the method of Goldblith & Proctor (1950). The units were based on the unimolecular reaction constant (Sumner & Somers, 1947).

The experiments were carried out in the Warburg manometric apparatus with flasks of about 20 ml. vol. at 38°, 5 min. being allowed for gassing and 10 min. for equilibration. The gas phase was oxygen, except where otherwise stated, and the volume of fluid in the flasks was 3 ml.

RESULTS

The succinoxidase system

In preliminary experiments the rate of oxidation of succinate by whole suspensions of muscle in 0·5% KCl solution was investigated and found to be

* Present address: Department of Biochemistry, University of Oxford.