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Measurement of the Arylsulphatase of *Patella vulgata* with 4-Methylumbelliferone Sulphate

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1. The preparation, purification, chemical and spectral properties of potassium 4-methylumbelliferone sulphate are described. 2. The use of 4-methylumbelliferone sulphate as a substrate for the arylsulphatase of *Patella vulgata* is presented with specific reference to the fluorimetric assay procedure used with this substrate. 3. 4-Methylumbelliferone sulphate is compared with the previously used synthetic sulphatase substrates nitrocatechol sulphate and *p*-nitrophenyl sulphate with respect to K_m, $V_{max.}$ and sensitivity in the assay of arylsulphatase. 4. 4-Methylumbelliferone sulphate was strongly inhibited by phosphate. Sulphate, a less potent inhibitor, appeared to be of the competitive type with some anomalous characteristics.

The compound MU* (4-methyl-7-hydroxycoumarin) is highly fluorescent in alkaline solutions. Substitution of the hydroxyl group causes the excitation wavelength of the umbelliferone to shift to shorter values and also brings about a decrease in the fluorescence. For these reasons, various conjugates of MU have been used for the assay of specific hydrolases. This technique utilizes the great differences in fluorescence between the conjugated umbelliferone and its hydrolysis product, the free umbelliferone, as a measure of the hydrolase activity.

Mead, Smith & Williams (1955) were the first to utilize this principle, by using 4-methylumbelliferone glucuronide to measure glucuronidase. These authors also described the preparation of MUS (potassium 4-methyl-2-oxo-1:2-benzopyran-7-yl sulphate) and suggested its use as a substrate for arylsulphatase; however, they considered it to be unsatisfactory because of its high blank values. Leaback (1961) synthesized several conjugates of MU including the sulphate and stated that MU could be measured in the presence of an excess of MUS. In the present paper it is shown that MUS is in fact a very sensitive and useful substrate for the measurement of arylsulphatase activity.

The arylsulphatase of the limpet *Patella vulgata* was chosen for the evaluation of MUS. This is a relatively simple enzyme that does not have the anomalous kinetics of the mammalian sulphatase A and that is, in addition, readily available, soluble and highly active. Further, since the *Patella* activity has been measured by means of substrates detected by standard absorptiometric techniques, MUS may be compared and contrasted with these. Thus the substrates APS, NCS and NPS have been used to study *Patella* by Dodgson & Spencer (1953), Roy (1956), Leon, Bulbrook & Corner (1960) and others.

A preliminary report of part of this work has appeared (Sherman, Allen & Stanfield, 1966).

* Abbreviations: MU, 4-methylumbelliferone; MUS, potassium 4-methylumbelliferone sulphate; NCS, dipotassium nitrocatechol sulphate; NPS, potassium *p*-nitrophenyl sulphate; APS, potassium *p*-acetylphenyl sulphate.

MATERIALS AND METHODS

Preparation of MUS. MUS was prepared by a variation of the method described by Mead *et al.* (1955). MU (Distillation Products Industries, Rochester, N.Y., U.S.A.) (10g.) was dissolved in anhydrous pyridine (20ml.). Chlorosulphonic acid (10g.) was added slowly to a second portion of anhydrous pyridine (30ml.) with stirring and cooling. After the addition of chlorosulphonic acid the pasty reaction mixture was brought to solution by warming. The pyridinium chlorosulphonate solution was then cooled to incipient precipitation and the solution of MU added in one portion. After standing overnight the mixture was diluted with water (200ml.) and made alkaline with $KHCO_3$. The MUS was precipitated, collected and crystallized from boiling water (charcoal). Samples of sufficient purity for use as substrate for fluorimetric assay were prepared by dissolving the MUS in a minimum of boiling water and adding a tenfold excess of ethanol. This procedure favours the precipitation of the MUS salt and dissolution of MU. The procedure is repeated until the ratio of fluorescence of MU to MUS is 4000 or greater at pH 10 with a filter fluorimeter prepared as described below. This was usually achieved in two or three crystallizations. Material obtained in this way was analysed by Micro-Tech

Laboratories, Skokie, Ill., U.S.A. (the sample was burnt in the presence of V_2O_5) (Found: C, 40·9; H, 2·4; S, 10·7. $C_{10}H_7KO_6S$ requires C, 40·9; H, 2·4; S, 10·9%).

This material gave a single spot, R_F 0·6, on thin-layer chromatography (silica gel G; E. Merck A.-G., Darmstadt, Germany) in butan-1-ol–ethanol–water (4:1:5, by vol.). MU gave a single spot at R_F 0·9.

The infrared spectrum was measured in Nujol mull on a Perkin–Elmer 237-B grating instrument. MUS differed from MU in having no absorption at 3150 cm.$^{-1}$ (lack of hydroxyl) and in having a strong absorption at 1300–1225 cm.$^{-1}$ characteristic of the sulphate ester.

In an attempt to obtain completely pure MUS, free of all MU, for fluorescence ratio studies, a sample was chromatographed by the thin-layer method and the MUS spot removed and eluted with pH 9 buffer. Contributions to the fluorimeter blank by the silica gel obscured whatever gain in purity was achieved by the chromatography. The material eluted did, however, have the same ultraviolet spectrum as the starting product (see Table 1) and was recovered in 93% yield.

A 1 mM solution of MUS is converted into MU (100% yield), as measured by fluorimetry, by heating at 80° for 30 min. in 0·3 N-HCl.

The upper limit of solubility of MUS in water or in 0·2 M-sodium acetate buffer, pH 5·4, is about 20 mM. To obtain a 20 mM solution it is necessary to warm the mixture at 37°. With concentrations below 15 mM solution is maintained even with freezing and thawing. Unbuffered solutions are stable for several months at −20°; however, a constant check on blank values is advisable as slow non-enzymic hydrolysis to MU occurs in samples that are in constant use (being thawed daily).

MU is completely stable under incubation conditions (Leaback & Walker, 1960) and standard aqueous solutions are similarly stable.

NCS (dipotassium 2-hydroxy-5-nitrophenyl sulphate) and NPS were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and used without further purification.

Enzyme. An acetone-dried powder of *Patella vulgata* was obtained from the Sigma Chemical Co. and used for all experiments. This is a partially purified preparation described by Dodgson & Spencer (1953) obtained by their simplified method. It is sold as a 'type III' preparation, and is essentially free of β-glucuronidase activity. The material used contained 0·36 mg. of protein/mg. of the enzyme powder by the method of Lowry, Rosebrough, Farr & Randall (1951).

The powder was made up as a stock solution containing 1 mg./ml. in 0·1 M-sodium acetate, pH 5·4, and stored at −20°. Freezing and thawing did not affect the activity.

Incubations containing less than 1 μg. of the *Patella* powder/0·2 ml., or incubations exceeding 10 min. duration, were carried out in the presence of 0·05% bovine serum albumin. This was found to stabilize dilute solutions, resulting in higher overall yields.

Assay procedure with MUS. In most experiments the buffered substrate plus enzyme and additions were incubated for 10 min. (except time-courses) in 0·2 ml. at 37° in 10 mm. × 75 mm. tubes. In all cases except the pH curve the incubations were carried out at pH 5·4 in 0·12 M-sodium acetate buffer. The reaction was terminated by the addition of 1·0 ml. of 0·1 M-2-amino-2-methyl-propan-1-ol hydrochloride, pH 10·3, and read in a fluorimeter prepared as described below. For maximum sensitivity the buffers and reagents must contribute a minimum of fluorescence to the system. This is achieved by using redistilled water for all solutions, and by treating the 2-amino-2-methyl-propan-1-ol hydrochloride solution with activated charcoal and filtering.

When working with limited amounts of enzyme, incubations were carried out in a volume of 0·02 ml. contained in 3 mm. × 60 mm. tubes. In this case the entire incubation mixture was pipetted, by a glass micro-pipette, directly into 1·0 ml. of the pH 10·3 buffer. The micro-pipette, which should be of approximately the same volume as the incubation mixture, is first filled with distilled water and then emptied in the usual way before the transfer. This procedure results in no more than a 2% loss of sample with a twofold increase in the standard error of the pipetting operation. Glassware is cleaned with hot alkali followed by hot HNO_3 with appropriate rinsing.

Appropriate controls were incubated with the samples to allow for non-enzymic hydrolysis of the MUS and to provide substrate blanks. In a 24 hr. incubation at pH 5·4 the fluorescence blank increased by about 25%.

Fluorescence was measured on an Aminco filter fluorimeter (American Instrument Co., Silver Spring, Md., U.S.A.). The most sensitive combination of light-source and filter was an 85 w General Electric mercury vapour lamp with a Corning 5860 as the excitation filter and a combination of Corning 5543 and 3387 for the fluorescence filter. The latter combination must be made with the 5543 towards the photomultiplier tube since the 3387 has a weak fluorescence at the wavelengths used. The fluorescence was measured directly in the 10 mm. × 75 mm. incubation tubes.

Assay procedure with NCS and NPS. Conditions were the same as those used by Roy (1956) and Leon *et al.* (1960). Both nitrocatechol and nitrophenol were measured on a Zeiss PMQII spectrophotometer (Carl Zeiss Inc., New York, N.Y., U.S.A.) at the appropriate wavelengths.

RESULTS AND DISCUSSION

The usefulness of MUS in the assay of arylsulphatase is derived from (1) its spectral properties and (2) its ability to react with the enzyme to release MU.

Spectral properties of MUS

As shown in Table 1, MU may be readily differentiated from MUS by fluorimetry at either alkaline or acidic pH values. Further, MU can be measured in the presence of MUS by absorptiometry at alkaline pH values.

When the fluorescence blank of the MUS buffered at pH 10·3 has been reduced to the minimum, as described in the Materials and Methods section, the range of measurement of MU in the presence of MUS extends to about 1 mμM by using fluorimetry. The fluorescence of MU is linear from 1 mμM to 1 μM and the extinction of MU is linear from 1 μM to 0·1 mM. Thus a wide range of concentrations are readily measured by utilizing fluorimetry or

Table 1. *Observed fluorescence and absorption maxima of 4-methylumbelliferone and 4-methylumbelliferone sulphate*

Fluorimetric data were obtained on an Aminco–Bowman spectrophotofluorimeter with an Osram 150w xenon arc. All data were corrected to mercury lines, but not for intensity of excitation light. Absorption maxima were determined on a Bausch and Lomb Spectronic 505. Buffers used were sodium phosphate and acetate.

	Absorption		Excitation	Fluorescence
	$\lambda_{max.}$ (mμ)	ϵ	$\lambda_{max.}$ (mμ)	$\lambda_{max.}$ (mμ)
pH 10·3				
MU	362	17000	359	451
MUS	362	<5	334	370
	311	10300		
	279	9720		
pH 4·0				
MU	320	13500	341	451
MUS	311	10500	334	379
	279	9860		

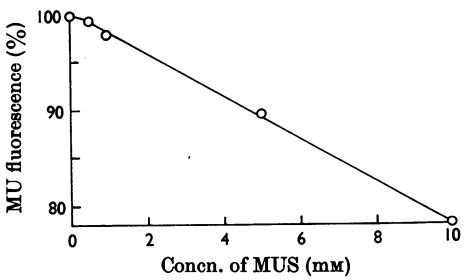

Fig. 1. Filter effect of MUS on the fluorescence of 0·3 μM-MU at pH 10·3. Samples were measured with a filter fluorimeter as described in the Materials and Methods section. The fluorescence of MU in the presence of 0–0·5 mM-MUS is 100±1%.

absorptiometry as the situation requires and excessive precautions need not be taken to have the results of an experiment fall within the capability of an instrument with limited range.

A further usefulness of MUS as a substrate is found in the ability to measure MU in the presence of MUS at acidic pH values by fluorimetry. Therefore the course of an enzymic reaction in the incubation mixture and at the desired pH may be followed, and a continuous record may be kept of the progress of reactions by using a chart recorder. This is especially useful for short time-course studies.

Table 1 shows that a discrepancy exists between the excitation and the absorption wavelengths of MU and MUS. This is a result of the lack of compensation for decreasing output of the xenon light-source of the fluorimeter below 360mμ. The apparent excitation maximum is due to a compromise between excitation light absorbed by the sample and that emitted by the xenon arc.

Fig. 1 shows that the absorption of excitation light by MUS (filter effect), which results in a decrease in the fluorescence yield of MU, is not appreciable until the concentration of MUS exceeds 0·5 mM. Below 0·5 mM the diminution of MU fluorescence is 1% or less. Thus for most cases no correction is necessary. In the extreme case of an incubation with 20 mM-MUS, a tenfold dilution to alkaline pH results in a 4% decrement in MU fluorescence.

MUS as a substrate

Effect of pH. The pH optimum of limpet arylsulphatase for 0·1 mM-MUS in 0·12 M-acetate buffers is 5·4, in agreement with the optimum found by Dodgson, Lewis & Spencer (1953) with APS, Roy (1956) with NCS and Leon *et al.* (1960) with NPS. *Patella* contains, in addition to arylsulphatase, both a steroid sulphatase (Roy, 1956) and a glycosulphatase (Dodgson & Spencer, 1954). We have obtained no direct evidence that would establish the degree to which MUS might be hydrolysed by these different sulphatases. With the steroid sulphatase, the pH optimum is 4·5 and the pH–activity plot of *Patella* with MUS shows no deviation from a smooth curve through the pH 4·5 region. Further, Roy (1956) presents evidence suggesting that NCS is not hydrolysed by the steroid sulphatase. The possibility remains, however, that MUS is hydrolysed to a degree by sulphatases other than arylsulphatase.

Effect of incubation time and enzyme concentration. An examination of the first 10 min. of incubation showed no deviation from a linear relationship between the extent of MUS hydrolysis and time. This continued to be linear for at least 3 hr. and decreased to about half the initial rate after 24 hr. The rate of hydrolysis was also found to be linear with respect to enzyme concentration in the range

0·6–18 μg. of *Patella* preparation/0·2ml. of incubation mixture. Beyond 18 μg. the activity fell and decreased rapidly between 30 and 60 μg./0·2ml. In 0·02ml. incubations linearity was observed between 0·0013 and 0·13 μg. Roy (1956) with NCS observed linearity with respect to time and enzyme concentration, whereas Dodgson *et al.* (1953) with APS found the rate to decrease over a 2·5hr. period.

Comparison of the effects on rate of varying MUS, NCS and NPS concentration. Fig. 2 shows the effect on the rate of hydrolysis of MUS, NCS and NPS by varying the concentrations of these substrates. Although MUS does not have the affinity for the enzyme that NCS displays, the rate is higher at all substrate concentrations measured. Each of these curves result in straight lines when plotted by the double-reciprocal method of Lineweaver & Burk (1934) (e.g. the uninhibited MUS in Fig. 3).

MUS has hydrolysis rates in the range 0·3–15mM

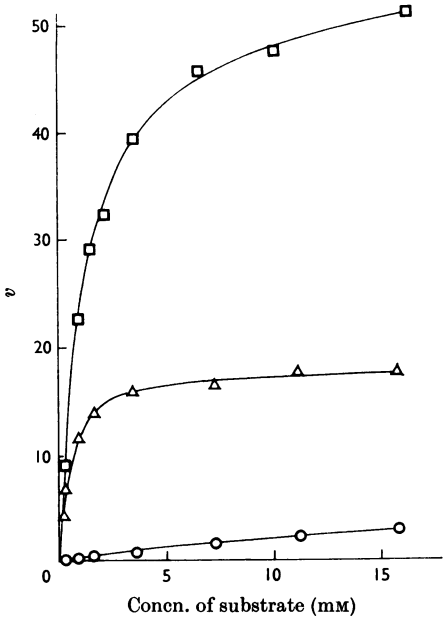

Fig. 2. Effect of varying substrate concentration on *Patella vulgata* arylsulphatase. For comparative purposes the experimental values have been normalized to rates that would be obtained with 1 μg. of the *Patella* preparation. The actual weights used are given. MUS (□) was incubated for 10min. in 0·2ml. of 0·12M-acetate buffer with 1·3 μg. of the *Patella*. NCS (△) was incubated for 30min. in 0·2ml. of 0·5M-acetate buffer, pH5·4, with 19·6 μg. of *Patella*; NPS (○) was incubated for 30min. in 0·2ml. of 0·5M-acetate buffer, pH5·4, with 205 μg. of the *Patella* preparation. Velocity, v, is expressed as mμmoles of product liberated/mg. of *Patella*/min.

that gave a straight line in the reciprocal plot. The apparent K_m for MUS from five determinations was 2·5 ± 0·5mM. The $V_{max.}$ for these experiments was 80 ± 10mμmoles/min./mg. of *Patella* preparation.

NCS was found to have an apparent K_m of 0·6mM and a $V_{max.}$ of 18mμmoles/min./mg. Roy (1956) obtained a K_m of 0·7mM for *Patella* with NCS.

NPS in two determinations gave an apparent K_m of 26 ± 2mM with a $V_{max.}$ of 8mμmoles/min./mg. This is considerably higher than the 6·6mM reported by Roy (1960). The reason for this is not known.

Comparison of MUS and NCS with respect to sensitivity of the method. Fluorimetric methods are generally considered to be about 1000 times as sensitive as absorptiometric ones. Under incubation conditions with *Patella* it is possible to detect reliably a minimum of 1mμM-MU. Under comparable conditions the minimum amount of nitrocatechol detectable is about 1 μM (an extinction of 0·01). Incubations under standard conditions with 15mM-MUS and with 20mM-NCS yield 38 and 17mμmoles/min./mg. of *Patella* respectively. It is thus seen that the potential sensitivity of the fluorimetric methods for the *Patella* arylsulphatase is about 2000-fold greater than the absorptiometric procedure. It should thus be possible to detect the activity of 0·05mμg. of *Patella*, by this method, with a 100min. incubation. This has not yet been realized in practice, however. In an experiment with 3·2mM-MUS, the smallest amount of *Patella* that could be measured in a 100min. incubation was 0·7mμg. When diluted to levels below 1mμg. in a 0·020ml. incubation volume the activity of the *Patella* rapidly diminished. This may have been due to denaturation or adsorption by glass surfaces and perhaps could be overcome by silicone treatment of the glassware used.

In an experiment comparable with the one described above, only incubating with 7·7mM-NCS, the smallest amount of *Patella* that could be measured was 130mμg. Thus the increase in sensitivity over NCS that has thus far been realized by using MUS is about 200-fold, tenfold less than that calculated from the known parameters.

Effect of chloride. Though Dodgson & Spencer (1953) and Roy (1956) observed activation of the *Patella* sulphatase at 10–50mM-chloride concentrations, we were unable to detect any effect with MUS in the range 1–500mM-sodium chloride.

Inhibition by phosphate. In common with other arylsulphatase substrates, the hydrolysis of MUS is strongly inhibited by phosphate. When 0·38mM-MUS is incubated with *Patella* in the presence of 0·1mM-potassium phosphate the fractional activity α, i.e. V_i/V, is 0·14 of the uninhibited activity. With the same phosphate concentration and with

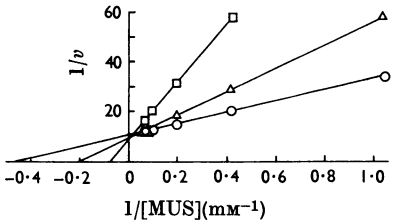

Fig. 3. Sulphate inhibition of MUS hydrolysis by *Patella* arylsulphatase. Concentration of K_2SO_4: (○) none; (△) 0·5mM; (□) 3·0mM. Velocity, v, is expressed as mμmoles of MU produced/1·28μg. of *Patella* preparation/min.

2·4mM-MUS, α is equal to 0·24. Attempts to establish whether the inhibition of MUS by phosphate is competitive or non-competitive have been inconclusive. All of the experiments where rates are examined at fixed phosphate concentration and varying MUS concentration have one thing in common: the fractional activity, α, increases with increasing MUS concentration. Plots of the kind described by Hunter & Downs (1945), where $I[\alpha/(1-\alpha)]$ is examined as a function of MUS concentration, give sloping lines. These lines differ from purely competitive (sloping) lines obtained by this method in that they do not converge at the $I[\alpha/(1-\alpha)]$ axis. They also differ by their slope from purely non-competitive (horizontal) lines. It is possible that this represents a mixed competition, although further evidence would be necessary to establish this.

Inhibition by sulphate. As with other substrates, MUS hydrolysis by arylsulphatase is inhibited to a smaller degree by sulphate than it is by phosphate. When MUS hydrolysis rates are measured in the presence of different concentrations of sulphate it is seen that the curve of the inhibited rate is asymptotic to 14% of the uninhibited rate beyond 10mM-potassium sulphate. This has been observed by Dodgson & Spencer (1953), who found that, whereas 10mM-sulphate inhibited the reaction by 43·7%, increasing the sulphate to 20mM only decreased the activity by 0·1%.

Double-reciprocal plots of MUS hydrolysis with and without added sulphate approximate closely to competitive inhibition. Fig. 3 shows the degree of deviation from simple competition. The uninhibited reaction in Fig. 3 intercepts the $1/s$ axis, to give a K_s to the normal range (2·15mM); however, the sulphate-inhibited reactions give two different values for K_i if one assumes perfect competitive behaviour and sets the intercept equal to $-1/K_s(1+I/K_i)$. The values obtained in this way are: 0·5mM-sulphate, K_i 0·38mM; 3·0mM-sulphate, K_i 0·54mM. The tendency for this apparent K_i to increase with increasing sulphate concentration has been observed repeatedly.

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