pancreatic lipase. This is not unexpected since these esters have a zwitter-ionic structure in contrast to the neutral structure of triglycerides. (3) Cholesteryl oleate is resistant to pancreatic lipase. It behaves, predictably, like an ester of a non-activated secondary alcohol[7]. (4) Rates of hydrolysis are not higher for medium chain-length esters than for oleic esters. Where higher rates have been reported[2–4], enzymes other than pancreatic lipase may have been measured. Medium chain-length esters appear, therefore, to be quite unsuitable for the detection or assay of pancreatic lipase. (5) The possibility remains that completely insoluble esters, like β-naphthyl oleate, though being rather poor substrates, will satisfy the requirement of specificity, *i.e.* will not be hydrolyzed by any enzymes except lipases. They could thus be used for quantitative determinations of lipases; however, the identity of the enzyme should always be verified by the hydrolysis of triolein or olive oil.

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4-Methylumbelliferyl phosphate as a substrate for lysosomal acid phosphatase

In their classical work on lysosomes APPELMANS *et al.*[1] used β-glycerophosphate as substrate for acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2). This gave good localisation of the enzyme in the lysosome fraction with a high degree of latent activity. Other workers[2–4] have used *p*-nitrophenyl phosphate for assaying the same marker enzyme, although it was shown by NEIL AND HORNER[5] and confirmed by BROTELLE AND WATTIAUX[6] that microsomal phosphatase is capable

of hydrolysing this substrate and is a possible contaminant of lysosome preparations.

The results of measurements of the integrity of the lysosomal membrane by assaying enzymes before and after potentiating the latent activity depend on the substrate used. Work in this laboratory has consistently shown better latency of β-glycosidases when 4-methylumbelliferyl substrates are used than when using nitrophenyl glycosides. This is probably due to the fact that the latter appear to penetrate the lysosomal membrane with ease[7]. 4-Methylumbelliferyl phosphate[8] has been used for the assay of alkaline phosphatase, and we have therefore compared this substrate with β-glycerophosphate and some commonly used aryl phosphate esters in acid phosphatase assays on the cell fractions obtained during the preparation of a lysosome enriched fraction from rat liver.

Lysosome-enriched fractions were prepared from livers of male Sprague–Dawley rats which were starved overnight before killing. The livers were rapidly removed and kept on ice until homogenised by hand in a Potter–Elvehjem type apparatus with a loose fitting Teflon plunger. Differential centrifugation of the homogenate in 0.25 M sucrose as described by DE DUVE *et al.*[9] gave nuclear, mitochondrial, lysosomal, microsomal particulate, and soluble fractions.

Cytochrome oxidase activity used as a marker for mitochondria and urate oxidase (peroxisome marker) were assayed as described by LEIGHTON *et al.*[10]. Protein was measured in a Technicon autoanalyser by a modification of the Folin method using casein as a standard.

All phosphatase assays were carried out in 0.05 M sodium acetate buffer (pH 5.0) at 37° for 10 min. The solutions were all made up in 0.25 M sucrose to preserve the integrity of the lysosomes and where total activities were required Triton X-100 was added to a final concentration of 0.1% (v/v). Enzyme preparations were also prepared and diluted to a suitable concentration for assay in 0.25 M sucrose. Standard conditions of assay were applied where available; β-glycerophosphate[1], glucose 6-phosphate[11], 5'-AMP[12] and phenyl phosphate[13] hydrolysis was followed by the assay of the liberated P_i as described by ALLEN[14], liberated *p*-nitrophenol was estimated colorimetrically[5]. 4-Methylumbelliferone liberated from its phosphate ester was assayed as follows.

To 1 ml of a suitably diluted enzyme solution or cell fraction was added 1 ml of freshly prepared 0.1 mM 4-methylumbelliferyl phosphate in 0.05 M acetate buffer. After incubation at 37° for 10 min the assay was terminated by the addition of 3 ml of 0.5 M glycine–NaOH buffer (pH 10.4). At this pH, maximum fluorescence of the liberated 4-methylumbelliferone is obtained and was measured in a Locarte LF 5 single-sided fluorimeter. The solution was excited by the 365-nm emission from a mercury lamp through an LF. 2 filter transmitting between 320 and 380 nm. The resulting fluorescence of the ionised 4-methylumbelliferone (λ_{max} 448 nm) was measured with the secondary beam monochromator set at 450 nm.

A fluorescence standard containing 4 μg/ml quinine in 0.05 M H_2SO_4 was used and the enzyme samples were diluted so as to liberate 0.5–5.0 nmoles 4-methylumbelliferone in 10 min. Under these conditions the solutions are clear (< 5 μg protein per ml) and no deproteinisation is necessary. Control experiments were performed in which the substrate was incubated in the absence of enzyme to compensate for non-enzymic hydrolysis the appropriate volume of enzyme being added after the glycine–NaOH buffer.

The results of a typical experiment are shown in Table I which indicates the

TABLE I

DISTRIBUTION OF ENZYME ACTIVITY ASSAYED IN THE PRESENCE OF 0.1% TRITON X-100 AFTER CELLULAR FRACTIONATION BY DIFFERENTIAL CENTRIFUGATION

Details of preparation of the fractions are given in the text. Activity is expressed as % of total activity occurring in any given fraction and in parentheses as relative specific activity = (%total activity in that fraction)/ (%total protein in that fraction).

Substrate	*Cell fraction*				
	Nuclear	*Mitochondrial*	*Lysosomal*	*Microsomal particulate*	*Soluble*
β-Glycerophosphate	10.6 (0.51)	17.9 (1.36)	45.6 (6.37)	16.1 (1.16)	9.8 (0.22)
4-Methylumbelliferyl-phosphate	17.2 (0.83)	15.2 (1.15)	44.0 (6.12)	14.8 (1.07)	8.8 (0.20)
p-Nitrophenyl phosphate	15.1 (0.71)	9.7 (0.74)	25.9 (3.6)	19.2 (1.40)	31.1 (0.70)
Phenyl phosphate	16.7 (0.79)	9.0 (0.68)	27.8 (3.85)	14.3 (1.04)	32.2 (0.72)
Glucose 6-phosphate	15.8 (0.75)	7.4 (0.56)	5.7 (0.79)	66.9 (4.83)	4.2 (0.09)
5'-AMP	37.5 (1.77)	10.4 (0.79)	16.1 (2.25)	36.0 (2.60)	0 (0)
Cytochrome *c* (oxidase)	16.1 (0.76)	61.0 (4.62)	16.1 (2.24)	6.8 (0.49)	0 (0)
Urate (oxidase)	8.4 (0.4)	3.8 (0.29)	84.0 (11.62)	3.8 (0.27)	0.(0)

proportion of the total measurable activity that is found in a given fraction when assayed by the methods described and also shows how the concentration of the enzyme is increased over the original homogenate (relative specific activity). The distribution of cytochrome oxidase and urate oxidase is as expected for their known cellular locations and 5'-AMPase shows the distribution of a membrane bound enzyme. The phosphatases show a spread of activity through all fractions and only β-glycerophosphate and 4-methylumbelliferyl phosphate give the high relative activity in the lysosome fraction that would be required for a good marker enzyme and show less than 10% of the total activity in the soluble fraction. Phenyl and nitrophenyl phosphatases on the other hand are detectable in the microsomal and supernatant fractions with almost one-third of the total activity in the soluble fraction and consequently a lower relative specific activity in the lysosomal fraction.

Table II shows how the observed latency of lysosomal acid phosphatase may depend on the substrate used. In this case a single lysosomal preparation was assayed with four different phosphates as substrates in the presence and absence of Triton X-100. The highest latency was observed when the fluorigenic 4-methylumbelliferyl phosphate was used and the lowest latency was observed with the nitrophenyl phosphate, even though its final concentration was only one-twentieth of that of the standard β-glycerophosphate.

It is thus clear that apart from the simplicity of the estimation, there is little to recommend the use of nitrophenyl phosphate in this context and results obtained with it are not comparable to those obtained with β-glycerophosphate. On the other hand the latter must normally be used at high substrate concentrations since the affinity of the enzyme for this substrate is not great, the assay of the liberated phosphate is somewhat tedious, and it is important that it is not contaminated with α-glycerophosphate which is readily hydrolysed by microsomal enzymes.

There are several advantages in using 4-methylumbelliferyl phosphate as a substrate in these assays, despite the fact that it is the least readily hydrolysed of all the substrates used. The results presented here show that the comparative values in

TABLE II

OBSERVED LATENCY OF LYSOSOMAL ACID PHOSPHATASE AS ASSAYED BY FOUR DIFFERENT METHODS

Final substrate concentration in parentheses. Latency is expressed as that proportion of the total activity that can only be detected in the lysosomal fraction after the incorporation of 0.1% (v/v) Triton X-100 into the incubation mixture. Specific activity is defined as μmoles substrate split per min per mg protein.

Substrate	*Concn. (mM)*	*Latency (%)*	*Specific activity*
4-Methylumbelliferyl phosphate	0.05	95.5	0.18
β-Glycerophosphate	50	92.8	0.89
Phenyl phosphate	50	76.6	1.70
p-Nitrophenyl phosphate	2.5	74.8	0.96

cell fractions are in close agreement with the results obtained by using β-glycerophosphate. A substrate concentration at least 1000 times lower can be used thus avoiding the introduction of high concentrations of Na^+ and even at this level the sensitivity of the fluorimetric method is much greater than that of the colorimetric assay. This is seen by the fact that the preparation had to be diluted a further 20 times before assay by the fluorimetric method.

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