Deficiency of Acid Esterase Activity in Wolman's Disease

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Young, E. P., and Patrick, A. D. (1970). *Archives of Disease in Childhood*, **45**, 664. **Deficiency of acid esterase activity in Wolman's disease.** Liver, spleen, and leucocytes from patients with acid triglyceride lipase deficiency (Wolman's disease and its clinical variants) were also found to possess greatly reduced activity of an acid esterase acting on fatty acid esters of p-nitrophenol, thereby substantiating the view that a single enzyme is responsible for these different activities. The acid esterase was resistant to the microsomal esterase inhibitor, E600, and showed broad specificity with respect to fatty acid chain length of the p-nitrophenyl esters. Other lysosomal hydrolase activities were increased non-specifically in liver from patients, thus providing further support for the classification of acid lipase deficiency as an inborn lysosomal disease. The highly sensitive leucocyte assay provides a convenient method for the diagnosis of clinical variants of Wolman's disease; it might therefore prove particularly useful in the early detection of affected infants, and also possibly in the differentiation of heterozygotes.

Wolman's disease is a rare inherited disorder of lipid metabolism in which large amounts of triglycerides and cholesteryl esters accumulate in the visceral organs. The main clinical features of the acute infantile form of the disease, typified by the cases first described by Wolman (Abramov, Schorr, and Wolman, 1956; Wolman *et al.*, 1961; Kahana, Berant, and Wolman, 1968) are failure to thrive, vomiting and diarrhoea, progressive hepatosplenomegaly and abdominal distension, radiological evidence of enlargement and calcification of the adrenals, and death in a severely wasted state before the age of 6 months. Similar cases have been reported by Crocker *et al.* (1965); Konno *et al.* (1966); Marshall *et al.* (1968), and Guazzi *et al.* (1968). However, it is our experience that cases also occur having biochemical and histological features identical to those of the acute infantile form of the disease, but which are of later onset and follow a much less severe and more prolonged course. In spite of this variation in clinical expression, the different forms of the disease are characterized by the same enzymic abnormality, namely, deficiency of an acid lipase acting on triglycerides and cholesteryl esters in tissues such as liver and spleen (Patrick and Lake, 1969). Furthermore, it

has been shown that lipid storage in these tissues is confined to intracellular bodies possessing histochemical and ultrastructural characteristics of lysosomes (Lake and Patrick, 1970). These findings suggest that the primary genetic defect in this condition is a deficiency of a lysosomal acid lipase, the natural substrates of which, triglycerides and cholesteryl esters, consequently accumulate within swollen lysosomes.

Several properties of the acid triglyceride lipases of rat liver and kidney (Mahadevan and Tappel, 1968) and of human liver (Young and Patrick, 1969, unpublished results) are identical to those of similarly located acid esterases which act on fatty acid esters of p-nitrophenol, suggesting that a single enzyme is responsible for these different activities, and that the alternative use of the highly sensitive acid esterase assay would be of value in the investigation of conditions involving acid lipase deficiency, particularly Wolman's disease. The present report describes this application.

Methods

Biopsy and necropsy (within 5 hours of death): tissue specimens were frozen immediately in solid CO₂ and stored at -25 °C. The leucocyte plaque was separated from fresh heparinized blood after centrifugation at

Received 30 April 1970.

1000 g for 5 minutes; after being washed and centrifuged twice with 1 ml. cold 0.9% NaCl and then twice briefly with 1 ml. cold water, the final leucocyte pellet was homogenized in cold water (0.5 ml. for 10 ml. of whole blood) and frozen and thawed three times. Recently, it has been found that homogenization in a 1% aqueous solution of Triton X-100 yields extracts with higher and more consistent levels of esterase activity. Enzyme activity and protein content (corrected when necessary for haemoglobin contamination) were determined on the clear centrifuged solution. Separation of leucocytes by sedimentation in dextran solution invariably led to almost total loss of esterase activity.

Measurement of p-nitrophenyl esterase activity under determined optimum conditions, and the preparation of substrate-Triton X-100 dispersions, were similar to the methods of Mahadevan and Tappel (1968). For liver and spleen, the reaction mixture contained 50 μ moles glycine-HCl buffer, pH 4.0; 3.75 μ moles p-nitrophenyl ester (routinely, palmitate; Koch-light Ltd.); 12.5 mg. Triton X-100, and 0.1 ml. of a 2% liver homogenate in 0.45 M sucrose (or 0.1 ml. of a 1% spleen homogenate) in a final volume of 0.75 ml. After incubation for 10 minutes at 37 °C., the reaction was stopped by the addition of 20% perchloric acid (0.25 ml.) and the mixture was allowed to stand for 5 minutes. After centrifuging, 0.5 ml. supernatant was added to 3 ml. M bicarbonate-carbonate buffer, pH 9.0 and extinctions were read at 400 m μ in 1 cm. cuvettes after $\frac{1}{2}$ -1 minute. Stearate, myristate, and laurate esters may be used as alternative substrates in the assay.

The assay mixture for leucocyte preparations contained, in a final volume of 0.75 ml., 50 μ moles glycine-HCl buffer, pH 4.0; 3 μ moles p-nitrophenyl palmitate; 25 mg. Triton X-100, and a volume of leucocyte extract containing approximately 100 μ g. protein. The mixture was incubated for 30 minutes at 37 °C. and treated as in the above assay of liver and spleen. Appropriate blank assays were run simultaneously.

Other lysosomal enzyme activities in liver were measured according to the references given: acid phosphatase, β -galactosidase, N-acetyl- β -glucosaminidase (Van Hoof and Hers, 1968); α -glucosidase (Patrick, 1965).

Case Reports

The four cases investigated had the characteristic histological and lipid analytical features of Wolman's disease. Also, acid lipase activity towards triglycerides and cholesteryl esters was undetectable in liver or spleen from the patients (these findings in three of the cases were given in an earlier report by Patrick and Lake (1969). Complete clinical histories of Cases 1 and 2 have been described elsewhere (Marshall *et al.*, 1968).

Case 1. This girl made poor progress from birth and was first admitted to hospital at the age of 6 weeks with diarrhoea, weight loss, hepatosplenomegaly, and abdominal distension. She remained in a wasted condition and required a blood transfusion for the correction of anaemia. Bone-marrow at that time contained large vacuolated histiocytes. X-ray examina-

tion at 10 weeks of age revealed enlargement and calcification of both adrenals and suggested a diagnosis of Wolman's disease. Death occurred at the age of 17 weeks and the diagnosis was confirmed by necropsy studies.

Case 2. Progress of this boy was normal until the age of 4 months when he began to pass frequent bulky yellow stools. Bouts of vomiting occurred and the abdomen became distended, with moderate enlargement of the liver. During the next 6 months the diarrhoea persisted; he had feeding difficulties and did not gain weight; the abdominal distension and hepatosplenomegaly increased and he became anaemic. Bone-marrow contained numerous large vacuolated histiocytes. A liver biopsy at the age of 11 months showed features similar to those of Wolman's disease. Deterioration continued and death occurred at the age of 14 months. The diagnosis was confirmed by necropsy studies.

Case 3. This younger brother of Case 2 has suffered intermittent episodes of vomiting from early infancy and was first admitted to hospital at the age of 12 months with severe diarrhoea and an enlarged tense abdomen. At 13 months the vomiting increased in frequency and severity and he passed frequent bulky fatty stools. His weight was 10.0 kg. at 11 months and 7.7 kg. at 15 months. A liver biopsy at the age of 13 months showed features identical to those found for Case 2. Now aged 3 years and 3 months, he has continued to have bouts of vomiting and diarrhoea, exacerbated by increased fat intake, but remains reasonably well on a fat-restricted diet.

Case 4. A girl made poor weight gains as a baby and at the ages of 2 months and 9 months had attacks of vomiting and diarrhoea; at these times abdominal enlargement was noticed by her mother. She was first admitted to hospital at the age of 2 years and 10 months, with general malaise and an enlarged liver, and was investigated for possible glycogen storage disease. The histology and lipid analysis of a liver biopsy at that time were consistent with the findings in Wolman's disease, though the triglyceride excess was less pronounced than usual. Now aged 8 years, she weighs 23.2 kg. and is 118 cm. tall; she enjoys good health and attends school, and has no apparent clinical abnormality other than moderate hepatomegaly.

Results

A marked deficiency of acid esterase acting on p-nitrophenyl palmitate in liver and spleen of patients with acid lipase deficiency is shown in Table I. Generally, the activities of necropsy specimens of control tissues were considerably greater than those of biopsy specimens, suggesting that post-mortem autolytic processes result in a marked activation of the esterase, perhaps through

its release from a membrane-bound configuration. Compared to the appropriate range of values for necropsy and biopsy specimens of control tissues,

TABLE I
*Acid Esterase Activity of Liver and Spleen**

Tissue	Subject	Range (and mean)
Liver		
Necropsy	Controls (5)	3.8-27.5 (18.2)
	Case 1	0.4
Biopsy	Controls (15)	2.1-10.9 (6.2)
	Case 3	0.2
	Case 4	Undetectable
Spleen		
Necropsy	Controls (2)	38.5, 46.2
	Case 2	4.0
Splenectomy	Controls (16)	15.0-46.5 (26.4)

*Values expressed as μ moles of p-nitrophenyl palmitate cleaved/min. per g. wet weight of tissue at 37 °C.

the activities found for the patients were approximately 10% or less, of the lowest values recorded. When preparations of tissues of control subjects and patients were mixed, the resultant activities agreed exactly with the sum of the activities of the separately diluted preparations, thus indicating that the low activity in the tissues of the patients was not due to the presence of an endogenous inhibitor nor to the absence of an activator.

The specificity of the acid esterase acting on different p-nitrophenyl esters is shown in Table II. Laurate, myristate, palmitate, and stearate esters were hydrolysed to about the same extent by control liver extracts, and this activity was shown to be due entirely to the lysosomal esterase by being unaffected after pre-incubation of the extracts with 10^{-5} M diethyl p-nitrophenyl phosphate (E600), at which concentration the microsomal esterase is almost totally inhibited. When either the caprylate or butyrate ester was used as substrate, the apparent increase of acid esterase activity in liver, both of

control subjects and patients, was shown to be due to the increasing contribution of the microsomal enzyme to the activity at pH 4. After pre-incubation with 10^{-5} M E600, the activity of control liver extracts towards p-nitrophenyl butyrate and p-nitrophenyl caprylate had fallen 82% and 67%, respectively, the residual activities then being similar to the values obtained for the higher esters. The activity in the liver of the patients towards the butyrate and caprylate esters was completely inhibited after treatment with 10^{-5} M E600. Evidence of the broad specificity of the acid esterase with respect to fatty acid chain length of the p-nitrophenyl esters was demonstrated more clearly for spleen (Table II). Activities towards the different esters of chain length C_4 to C_{18} were similar and were unaffected by pre-incubation with 10^{-5} M E600. Furthermore, in contrast to liver, the activity in the spleen of Case 2 remained at a low level when the butyrate or caprylate ester was used as substrate, indicating that the microsomal enzyme made no contribution to the overall activity at pH 4.

Deficiency of acid esterase activity in the leucocytes of Cases 3 and 4 is shown in Table III. The mean activities increased considerably with the age of control subjects, the value for cord blood being only 20% of that of adults, so that it is advisable to restrict comparisons of activity in children to subjects of closely similar age. Acid esterase activities of leucocytes from both parents of Case 3 and from the mother of Case 4 were intermediate between the values for adult controls and those of the affected children, suggesting that the esterase assay might be of value in the detection of the carrier state in adults.

With a view to the prenatal detection of Wolman's disease, the acid esterase assay was also applied directly to normal amniotic fluid after 14 weeks of pregnancy. Detection of the esterase was uncertain using p-nitrophenyl esters, but preliminary tests

TABLE II
*Hydrolysis of Different p-Nitrophenyl Esters by Liver and Spleen**

Tissue	Subject	Butyrate	Caprylate	Laurate	Myristate	Palmitate	Stearate
Liver							
Necropsy	Control	61.2	18.8	16.5	14.0	17.5	15.3
	Case 1	31.3	6.3	1.8	1.5	0.4	0.5
Biopsy	Control	45.0	8.3	5.0	4.5	6.0	6.2
	Case 3	13.5	1.3	0	0.3	0.2	0.6
Spleen							
Necropsy	Control	40	36	36	39	39	40
	Case 2	0.8	1.5	2.0	—	4.0	—

*Values expressed as μ moles of substrate cleaved/min. per g. wet weight of tissue at 37 °C.

TABLE III
*Acid Esterase Activity of Leucocytes**

Subject	Range (and mean)
Controls:	
Adult (16)	30.3-83.5 (54.8)
Children, ages 6-12 years (10) ..	18.7-82.5 (42.0)
ages 2-5 years (9) ..	22.2-41.6 (31.4)
ages 1 week-7 months (8)	16.7-32.3 (23.0)
Cord blood (8)	6.5-16.7 (11.2)
Case 3 (age 3 years)	4.3
Mother	24.8
Father	27.8
Normal brother (age 8 years) ..	24.4
Case 4 (age 8 years)	Undetectable
Mother	19.6

*Values expressed as μ moles of p-nitrophenyl palmitate cleaved/min. per mg. of protein at 37 °C.

have shown this to be possible using 4-methylumbelliferyl esters.

The activities of other lysosomal enzymes in liver from the patients are shown in Table IV. The results for the three cases studied suggested that the activities of α -glucosidase, acid phosphatase, and N-acetyl- β -glucosaminidase were individually or severally increased to a moderate extent, as compared to controls, while the activity of β -galactosidase in two cases was decreased.

TABLE IV
*Activity of Lysosomal Enzymes in Liver**

	α -Glucosidase	Acid Phosphatase	N-Acetyl- β -Glucosaminidase	β -Galactosidase
Mean for 8 controls	0.088	2.04	1.68	0.63
Range (% of mean)	71-128	48-151	70-141	67-146
% of mean control activity				
Case 1	165	167	144	86
Case 3	133	183	144	46
Case 4	165	162	178	44

*Values expressed as μ moles of substrate cleaved/min. per g., wet weight, of liver at 37 °C.

Discussion

Evidence of the single identity of a lysosomal esterase acting on p-nitrophenyl esters and a lysosomal lipase acting on triglycerides in rat and human tissues is substantiated by the finding of a marked deficiency of acid esterase activity in the tissues of patients having the histological and biochemical features of Wolman's disease, and in whom an acid lipase acting on triglycerides and cholesteryl esters was undetectable in liver and spleen (Patrick and

Lake, 1969). It is not known whether the low level of p-nitrophenyl esterase activity found in the tissues of patients is due to residual acid lipase resulting from an incomplete enzyme defect, or whether it derives from a different esterolytic enzyme, perhaps of an entirely non-specific character. Whatever the nature of this activity it is possible that it effects the slow intra-lysosomal hydrolysis of stored lipids and accounts for the appreciable increase of free cholesterol and fatty acids in the tissues of patients with acid lipase deficiency. In contrast to the acid esterases of rat liver and kidney, which are most active on the higher p-nitrophenyl esters (Mahadevan and Tappel, 1968), the acid esterase activities of human liver and spleen were similar for a range of esters of fatty acid chain length C_4 to C_{18} . This broad specificity of the human liver esterase has also been demonstrated by the use of α -naphthyl acetate and butyrate as well as α -naphthyl palmitate as substrates for the histochemical detection of a deficiency of E600-resistant acid esterase activity in liver from a patient with acid lipase deficiency (Lake and Patrick, 1970).

The greater sensitivity and simplicity of the p-nitrophenyl esterase assay compared to that of the acid triglyceride lipase assay permits accurate determinations to be made on small amounts of tissue, including leucocytes obtained from as little as 1 ml. blood. The use of leucocytes is an obvious advantage in the confirmation of a diagnosis in the acute infantile form of the disease, where surgical biopsy could not be undertaken, and provides a means of diagnosis at the earliest possible time after birth. Acid esterase activity of leucocytes develops slowly during infancy and childhood, the level in cord blood being only a small fraction of the adult level; nevertheless, the deficiency of acid esterase in Wolman's disease appears to be sufficiently well defined to allow certain diagnosis in early infancy, and possibly extends to the use of cord blood as assay specimen. A possible further application of the leucocyte esterase assay in the detection of the carrier state of Wolman's disease is suggested by the intermediate activities of leucocytes of parents of affected children, a finding that agrees with the concept, derived from a knowledge of the incidence of the disease, of an autosomal recessive mode of inheritance.

Electron microscopical and histochemical studies (Lake and Patrick, 1970) have demonstrated the site of lipid storage in liver from a patient with acid lipase deficiency to be within intracellular bodies having the characteristics of altered lysosomes, and it is assumed that the increase in the levels of several lysosomal hydrolases reported here occurs

at these sites. These non-specific changes accentuate the deficiency of acid esterase and substantiate the view that acid lipase deficiency should be classified as an inborn lysosomal disease. No explanation can be offered for the low activity of β -galactosidase in liver from the patients, but this finding suggests that some caution may be necessary in the interpretation of a non-specific partial deficiency of this enzyme in other lysosomal diseases.

The 4 patients studied here were affected by the same disease, as defined by a total lack of acid lipase acting on triglycerides and cholesteryl esters, and it is concluded that this deficiency reflects the primary genetic mutation from which secondary effects arise. Such secondary effects as histological changes and the accumulation of triglycerides and cholesteryl esters in the tissues were similar in the cases studied, yet there was great variability in their clinical manifestations. The features of the disease in Case 1 were identical to those described by Wolman, while Cases 2 and 3 showed variability in the same sibship with respect to age of onset, severity of symptoms, and duration of the disease; Case 4, alive and well at the age of 8 years, shows no clinical abnormality other than moderate hepatomegaly. Clinical variants of inherited metabolic disorders are being recognized with increasing frequency and present consequent difficulties to a diagnostic classification based on clinical manifestations. It therefore seems appropriate to use the general term 'acid lipase deficiency' to describe the lipodosis of which the acute infantile form first described by Wolman is a clinical type, for which the

designation 'Wolman's disease' is a suitable euponym.

We thank Dr. June Lloyd and Dr. D. G. Cottom for permission to study their patients, and the British Tay-Sachs Foundation for financial support.

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