ARGINASE ISOENZYMES IN HUMAN DIPLOID FIBROBLASTS.*

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Arginase activity has been demonstrated in cultured diploid fibroblasts and compared with that of the known arginases in other human tissues. In liver, kidney, erythrocytes and fibroblasts the ratios of specific activities are 750/45/45/1 respectively. Enzyme kinetics, pH-optimum, K_m-values and Mn^{++} effect are similar in all tissues. The known DEAE isoenzymes A_1, A_3 and A_4 also exist in fibroblast strains, which consistently show either A_1 and A_3 (liver pattern), or A_1 and A_4 (kidney pattern) or the mixed pattern A_1, A_3 and A_4.

INTRODUCTION.

Hyperargininemia has been reported in humans in only one family and was correlated with a deficiency of arginase (E.C.3.5.3.1) in erythrocytes (1). This observation has been the basis of a model for both in vivo and in vitro treatment of hereditary disease (2). An investigation of the in vitro phenotypic effect of the hyperargininemia causing mutation has yielded a simple method for the assay of arginase activity in cultured fibroblasts. This method presented below has allowed a comparison of some kinetic and DEAE cellulose chromatographic properties of the fibroblast enzyme with that in liver, kidney and erythrocytes.

MATERIALS AND METHODS.

1. Tissues and homogenates.

Fibloblast strains established from skin biopsies, were propagated until confluency in Ham's F10-medium (GIBCO H12) supplemented with 15% fetal calf serum (Flow Labs. N° 4-055E) and with antibiotics. Following thorough rinsing in saline the cells were harvested by scraping, centrifuged and kept at -20°C.

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Contamination by mycoplasma was tested by the assay of arginine-deiminase activity (3).

Post-mortem samples of human liver and kidney were thawed immediately before use. Homogenates of liver, kidney and fibroblasts were prepared in water 1/5 (w/v) with a glass-teflon homogenizer followed by sonication (1 min., 40 Watts, Branson sonifier).

Erythrocytes were prepared from heparinized blood, washed repeatedly in saline, centrifuged and thus stored at -20°C. They were simply lysed in an equal volume of water.

2. The arginase assay is a modification of Schimke's method (4).

The fibroblast homogenates were used as such. The original 1/5 organ homogenates were diluted in water (1/1000 : liver; 1/10 : kidney). Stock erythrocyte lysates were diluted 300 times. All homogenates were preincubated at 55°C for 20 min. in 20 µM glycine-NaOH buffer pH9.7, and a mixture of 0.3 µM $MnCl_2$ and 0.75 µM maleic acid pH 7.0. True incubation at 37°C was started by the addition of L-arginine pH 9.7 (12 µM fibroblasts; 20 µM : organs and erythrocytes). The enzymatic reaction was stopped by adding 200 µl of 0.5 M $HClO_4$. The precipitate was removed by centrifugation. Urea content in the supernatant was determined by the thiosemicarbazide-diacetylmonoxime method (5). Two kinds of blanks were carried : a zero time blank and a substrate blank, where the enzyme was substituted by distilled water.

3. DEAE cellulose chromatography (6).

Here appropriate homogenate dilutions for liver, kidney, ery-

Table 1.

Arginase activity in human tissues
(nM urea/hour/mg protein)

Tissue	N	Mean	SD	SEM	Range
Fibroblasts	5	82	44	20	20 - 141
Liver	8	58,120	33,850	11,960	31,000 - 117,000
Kidney	7	3,620	860	320	2,567 - 5,350
Erythrocytes	4	3,470	1,030	510	2,100 - 4,420

throcytes and fibroblasts were 1/250, 1/100, 1/100 and 1/5 respectively. 1 ml Homogenates were prepared in a 5 mM Tris-HCl buffer pH 8.3 containing 4 mM $MnCl_2$ and 10 mM maleic acid. They were cleared by centrifugation. The elution of the 8 mm x 26 mm DEAE cellulose (Whatman DE 52) columns was started with the 5 mM Tris-HCl equilibration buffer pH 8.3 and continued with a linear KCl gradient (0-0.3 M). 25 Fractions were collected. 4. Protein was measured according to Lowry's method (7).

RESULTS.

The conditions described above are optimal for the assay of human arginase and its specific activity (nM urea/hr/mg prot.) in different tissues is reported in Table 1.

Reaction kinetics of the enzyme are linear for more than two hours. The rate of urea production is also linear for amounts of fibroblast homogenate up tot 100 µl. The pH optimum is 9.7. An

Table 2.

Tissue	pH-optimum	K_m in µM
Fibroblasts	9.7	0.48
Liver	10.0	1.25
Kidney	9.7	1.05
Erythrocytes	9.7	0.91

Table 3.

Rates of product formation.

Tissue	nM urea/30 min	nM ornithine/30 min.
Fibroblasts	24	25
Liver	106	110

Table 4.

Arginase activity in nM urea/hour
in different conditions of assay

μM of Mn^{++}	0.0		0.3	
Preincubation	no	yes	no	yes
Fibroblasts	8.0	4.5	8.0	27.5
Liver	0.0	0.0	33.0	88.0
Kidney	0.0	0.0	80.0	240.0
Erythrocytes	0.0	0.0	10.0	37.5

arginine concentration of 12 μM provides optimal activity.

pH Optima in all tissues and apparent K_m-values derived from Lineweaver-Burk plots are presented in Table 2.

As a test for both the identity of the enzymatic activity and its method of assay the rate of simultaneous ornithine formation was measured in separate experiments. For this purpose the glycine-NaOH buffer was substituted by a carbonate buffer pH 9.7 (0.25 μM in the reaction mixture). Ornithine was determined by the ninhydrin method (8). As shown in Table 3, equimolecular amounts of urea and ornithine are produced from arginine.

The known activating and stabilizing effect of Mn^{++} on mammalian arginase was investigated in two types of experiments. 1/ Activating effect : arginase was assayed in homogenates either in the presence or in the absence of $MnCl_2$-maleic acid mixture with or without preincubation at 55°C. $MnCl_2$ activates optimally in quantities between 0.15 μM and 0.6 μM. The results are in Table 4. 2/ Stabilizing effect : homogenates prepared either in $MnCl_2$-maleic acid mixture or in maleic acid only (pH 7.0) were kept at 70°C. Aliquots were taken after 0, 15, 30 and 45 minutes for arginase assay. For results see Fig. 1.

DEAE ion-exchange fractionation results are presented in Fig 2. Using Porembska's terminology (6), fraction A_1 eluted with 5 mM Tris-HCl buffer is present in liver, kidney, erythrocytes and nine fibroblast strains examined. Fraction A_3 is elu-

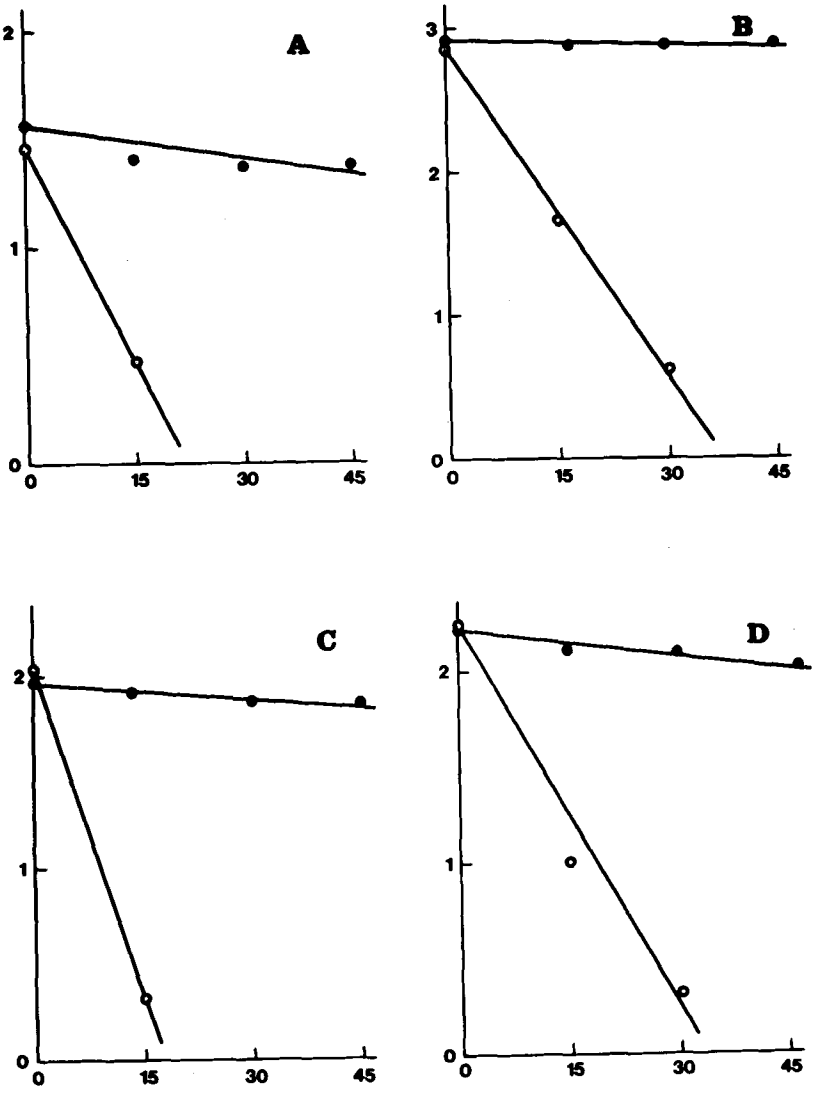

Fig. 1 Thermal inactivation of arginases in (A) fibroblasts, (B) liver, (C) kidney, (D) erythrocytes. Mn^{++} present (full circles), absent (open circles). Abscissa : time in min. Ordinate : log. nM urea/hr.

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ted with 0.08 M KCl. It is found in liver and in some fibroblast strains (i.e. strain 160). Enzyme A_4 eluted with 0.18 M KCl appears specifically in kidney. It is present in other cell strains such as 168. Still other fibroblast strains have all three types of arginase (A_1, A_3 and A_4). Strain 106 is the example in Fig. 2. Cochromatography of equal amounts of the homogenates 160 and

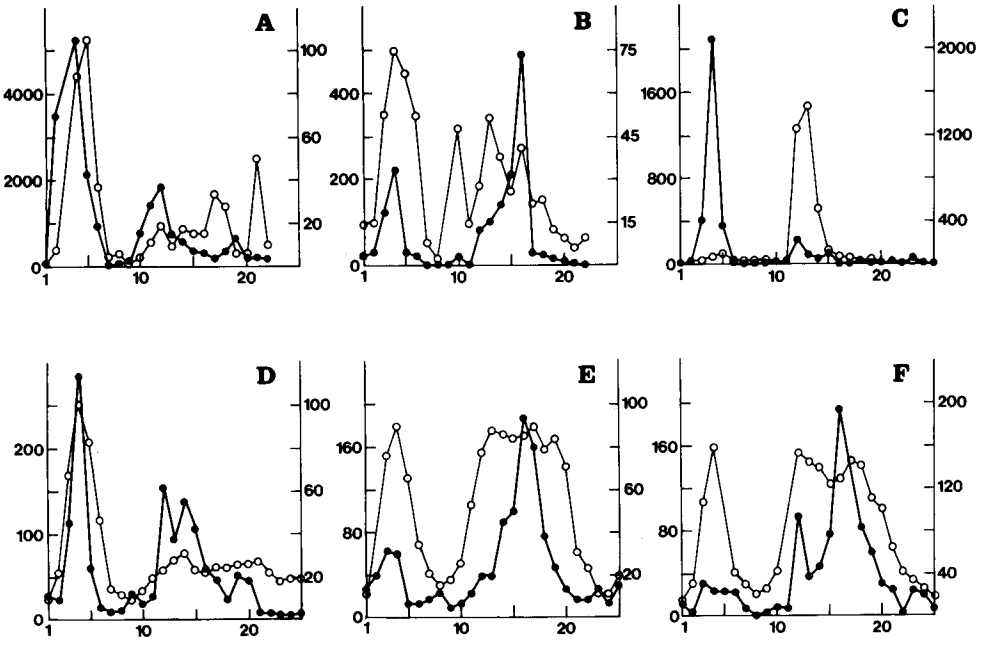

Fig 2 DEAE ion-exchange chromatography of arginases in human liver (A), kidney (B), erythrocytes (C) and the fibroblast strains 160 (D), 169 (E) and 106 (F). Abscissa : fraction number. Ordinates : left sides : arginase (full circles) in nM urea/hr; right sides : protein (open circles) in µg.

168 yields the expected elution pattern with all three arginase fractions.

DISCUSSION.

In studying citrullinemia Tedesco and Mellman observed that only the citrulline to arginine portion of the urea cycle exists in human diploid fibroblasts (9). Previous experiments in our laboratory have also shown that propagation of such strains cannot be maintained in selective culture media wherein either citrulline or arginine were substituted by ornithine. Enzymes catalyzing the citrulline to arginine part of the urea cycle were demonstrated in cultured cells by several investigators (9-11). In all those studies arginase as well as ornithine transcarbamylase were thought to be absent.

Our results prove that arginase is functional indeed in diploid fibroblasts.

The identity of the enzyme is shown satisfactorily by three

lines of evidence. Firstly, although the considerably lower specific activity in fibroblasts requires less substrate for optimal assay, enzyme kinetics are similar to those of the arginases in erythrocytes and other tissues. Secondly, the enzyme hydrolyzing arginine yield equimolecular amounts of urea and ornithine. Thirdly, DEAE ion-exchange chromatography of fibroblast homogenates separates fractions similar to those obtained in other tissues (6).

The weak activity of arginase in fibroblasts is expected because their urea cycle is incomplete. Contamination by arginine deiminase positive Mycoplasmas must be avoided. The pH-optimum of their enzyme is 6.8, but enough citrulline is formed at pH 9.7 in contaminated homogenates to interfere with the photometric method used for determination of urea and ornithine.

Addition of Mn^{++} activates and stabilizes the arginases in all tissues. However the importance of an activating effect by Mn^{++} upon crude non-dialyzed stock homogenates of fibroblasts remains unclear.

From the repeated chromatographic study of nine different cell homogenates, it appears that fibroblasts can be divided in three categories with respect to the arginase isoenzymes present. One group shows the isozymes A_1 and A_3 also found in liver homogenates. Another group of strains likewise contains the A_1 isoenzyme but in addition the form found in kidney and called A_4 by Porembska (6). Finally in a third group of homogenates, A_1, A_3 and A_4 are all present.

It is unlikely for several reasons that all arginase in fibroblasts is derived from uptake from the serum (12) in the culture medium : 1/ Chromatography of fetal bovine serum shows that all arginase (60-80 µM urea/ hr/ml) is only in a single peak (eluted by 0.13 M KCl). 2/ No significant difference of specific activity is noted in fibroblasts grown for 2 months in either fetal calf serum or newborn calf serum containing medium. The latter contains about 50 times less arginase; 3/ Arginase activity in culture medium remains constant throughout six-day periods of cell cultivation.

Either of the three isoenzyme patterns observed is found consistently in any given cell strain. As the fibroblasts were always harvested in the stationary phase of growth change of isoenzyme pattern with phase of in vitro propagation cannot

be ruled out. Such change of pattern has in fact been observed for another enzyme (13).

This quantitative and qualitative information on arginase has provided an excellent opportunity to study the phenotypic expression in fibroblasts of the hyperargininemia causing mutation in humans. The results obtained shall be reported shortly.

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