IUGR pups showed significantly higher total plasma amino acid levels at birth, compared to control pups of normal body size, with particular increases in gluconeogenic amino acids (alanine, glycine, proline, and valine). A fall to low levels of circulating amino acids was observed at 2–4 hr in the untreated progeny, coincident with the development of hypoglycemia. The group administered corticosteroids prenatally had more pronounced increases of plasma gluconeogenic amino acids than untreated animals, with alanine especially elevated. Despite 3-fold greater apparent utilization in animals administered glucocorticoid prenatally, high alanine levels were maintained in the treated dysmature group; a similar pattern was noted for glutamine-glutamate. In addition, an inverse correlation between plasma glucose at birth and plasma alanine levels was noted for all animals ($r = -0.55, P = 0.01$), as well as an inverse correlation between plasma glucose and the four gluconeogenic amino acids ($r = -0.62, P = 0.001$).

Thus, the reduced tendency to develop hypoglycemia in corticosteroid-treated dysmature rat pups seems to be associated with both greater availability and increased utilization of glucogeneic amino acids. We therefore postulate that the hormone may act both to increase the supply of gluconeogenic substrate and to stimulate enzymes essential for gluconeogenesis.

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Arginine Esterase and Lysosomal Hydrolases in Liver from Cystic Fibrosis Subjects*

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Summary

The total activity and isoelectric focusing patterns of arginine esterase, cathepsin B_1, and several lysosomal hydrolases were normal in liver from two patients with cystic fibrosis. No abnormalities were observed in values for pH optimum, K_m, and V_{max} for arginine esterase and cathepsin B_1 in liver from cystic fibrosis patients compared to those values for liver from the control subject. Soybean trypsin inhibitor at concentrations up to 100 μg/ml had no effect on liver arginine esterase or cathepsin B_1.

Speculation

The data suggest that none of the enzymes studied in this work is directly related to the genetic defect in cystic fibrosis. Liver arginine esterase is different from the major arginine esterase found in plasma and saliva but may be identical to the minor component. The reported deficiency (12–15) of the major plasma arginine esterase may be a secondary phenomenon resulting from inadequate exocrine (chiefly pancreatic) secretion.

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INTRODUCTION

Cystic Fibrosis is an autosomal recessive condition characterized by malfunction of exocrine tissues (7). The genetic defect in this disease has not been determined to date. Several recent reports have suggested the involvement of proteolytic enzymes in the disease process (10,12,14-17). Other investigators have demonstrated the presence in CF serum of a low molecular weight basic protein (3,8-10). This protein had the capacity to disorganize rhythmic beating of the cilia. It was later found that the action of this factor could be destroyed by treatment with the enzyme carboxypeptidase (10). This suggested the presence of functionally important arginine residues in the protein. These data correlate well with recent reports by Nadler and his associates who have postulated a role for an arginine-esterolytic enzyme in the pathology of this disease (14-17). The enzyme, arginine esterase, is active with the synthetic substrate α-N-benzoyl-L-arginine ethyl ester (BAEE). Multiple forms of this enzyme have been shown in human plasma by means of ion exchange chromatography, isoelectric focusing on polyacrylamide gels and by inhibition with soybean trypsin inhibitor. Rao and Nadler (14-16) found that in plasma from CF patients there was an almost total deficiency of the arginine esterase activity susceptible to inhibition by soybean trypsin inhibitor. In a more recent report, these authors confirmed a deficiency of arginine esterase in CF plasma compared to controls with protamine as substrate (15). In the present study, we have measured the activity of arginine esterolytic and other hydrolytic enzymes in liver to determine if the deficiency of arginine esterase observed in plasma and saliva was also generally true in other tissues. A preliminary communication of this work has been published (6).

METHODS AND MATERIALS

CASE MATERIAL

Liver was obtained from two CF patients NL (male, 25 years, number 1 in Table 1) and BY (female, 19 years, number 2 in Table 1). Both had been investigated in this hospital for many years. NL was diagnosed at age 2 and recent history included respiratory failure. At post mortem, there was extensive bronchiectasis, hepatomegaly, splenomegaly, right ventricular hypertrophy and a fibrotic pancreas with no cysts. Liver function tests were normal. The liver was obtained 19 h after death.

BY from a family of five siblings, four of whom have documented Cystic Fibrosis, was diagnosed at the age of 8 years. Her clinical course was characterized by recurrent respiratory infections. She developed cor pulmonale and on her terminal admission sustained three cardiac arrests, the last of which was fatal. There was fibrosis of the pancreas with cysts and with destruction of exocrine tissue. A severe bronchiectasis was found and the liver did not show longstanding passive congestion. The liver was taken 5 hours after death.

Control specimens were from patients who died at the age of 8,15 and 18 years who had no evidence of liver dysfunction. The patient with Type I, G_{M1}-gangliosidosis died at the age of 2 years while the patient with Gaucher disease died at 8 years. Lipid storage and enzymopathies have been confirmed in the latter two cases.

MATERIALS

Hemoglobin (Type 1, bovine blood), Fast Garnet-GBC salt, p-tosyl-L-arginine methyl ester - HCL (TAME), N-Carbobenzoxy-α-L-glutamyl-L-tyrosine, bovine serum albumin, Benzoylarginine-β-naphthylamide (BANA) Soybean trypsin inhibitor (type 1-S, lot 85C-8055, with a stated potency of 1 mg/1.6 mg Trypsin), (23), N-Benzoyl-arginine ethyl ester (BAEE, lot 072074) (24) and lyophilized Trypsin (198 units/mg) (25) were obtained commercially.

TISSUE EXTRACTION

No specimens had been stored longer than 12 months. All tissues were frozen at -20°C until processed. Several specimens from each tissue (0.5-0.7 g) were taken over several months. The samples were minced with scissors and homogenized in 10 volumes of 0.05 M sodium acetate-acetic acid buffer containing 1 mM EDTA and 0.154 M sodium chloride (final pH 4.6). The extract was centrifuged at 31,500 g for 30 min. at 2° in a Beckman J-21 centrifuge. The supernatant fluids were then stored at -20° until processed.

ENZYME ASSAY

Cathepsin A was measured with N-CBZ-glutamyl-tyrosine substrate while cathepsin D was measured with acid denatured hemoglobin (11). Cathepsin B_1 was assayed with BANA substrate according to Barrett (2). Arginine esterase was measured by two methods. The first according to Rao and Nadler (16) was modified as follows: each quartz cuvette (1 cm light path) contained in 1 ml final volume, 100 μmoles Tris-HCl pH 9.0 and 0.5 μmoles BAEE. The reaction was started by addition of the enzyme extract after a 5 minute pre-incubation period. The temperature of the cuvettes was maintained at 37.0 ± 0.5°C by circulating water from a Lauda K-2 thermostat. The initial velocities were calculated from the slope of the line measured at 253 nm. over the first 3-5 minutes. The second assay for arginine esterase utilized the TAME substrate according to Siegelman et al. (20)

The glycosidases, α-mannosidase, α-fucosidase and β-hexosaminidase were assayed according to Thomas et al (21) while G_{M1}-ganglioside β-galactosidase was assayed according to Callahan and Gerrie (4). Sphingomyelinase was assayed according to the method of Schneider and Kennedy (19). Protein measurements were made with bovine serum albumin as standard according to the method of Lowry et al. (13)

Assays performed to determine the total activities of the various hydrolases in the liver extracts were carried out at five protein concentrations at a single time point. The progress of the reactions with time was linear in every instance. The units of activity and specific activities are defined in the legend to Table 1.

ISOELECTRIC FOCUSING

Isoelectric focusing was carried out as described previously (5). Briefly, tissues 0.5 - 0.7 g were minced with scissors and homogenized in 10 vol 0.05 M sodium acetate-acetic acid buffer containing 1 mM EDTA (final pH 4.6). The extract was centrifuged as above and the resulting fluids were exposed to current for 40-44 hours in a thermostatically controlled column. The experiment was carried out in the presence of sucrose gradients with 1% carrier ampholines, pH range 4-7. At the end of the experiment the column was eluted from below and the eluates were collected in 0.8 - 1.0 ml fractions. All eluates were kept at 4° C until analyzed. All enzymes were assayed in the presence of carrier ampholines and sucrose.

RESULTS

Liver samples from the two controls, the two CF patients, and single cases of G_{M1}-gangliosidosis and Gaucher Disease were examined (Table 1). In the liver of one CF patient the total activities of all enzymes were within control values. All enzyme activities were elevated in the liver from the second CF patient except for β-hexosaminidase, α-mannosidase and α-fucosidase. The elevations seen in the lysosomal hydrolases measured in liver from Gaucher Disease and G_{M1}-gangliosidosis are consistent with previously published data (22).

Cathepsin B_1 was found to have a maximum activity at pH 6.4 in both normal liver and in liver from patients with Cystic Fibrosis. The activity in the presence of cysteine was higher than the activity in its absence in all buffers tested and throughout the pH range (Fig. 1). There was no difference in the Km or Vmax values for cathepsin B_1 in normal and Cystic Fibrosis liver (Table 2). Soybean trypsin inhibitor was tested for its effect on the activity of cathepsin B_1. No inhibition of cathepsin B_1 activity could be demonstrated over the range of 5-100 μg. Under the same conditions trypsin (1 μg) was completely inhibited at the level of 6 μg.

There was no difference in the level of arginine esterase between three normal controls and the liver samples from patients with Gaucher Disease and G_{M1}-gangliosidosis (Table 3). On the other hand, with BAEE there was a 50 per cent decrease in the total level of arginine esterase in the two patients with Cystic Fibrosis. With TAME as substrate however, there was little or no difference between the levels of the enzyme in the normal and the CF specimens. The Km and Vmax values for this enzyme were unchanged from normal (Table 2). The pH optimum of arginine esterase with both substrates was in the range of 8.5 - 9.1 for both normal and CF liver specimens (Figure 2).

The effect of soybean trypsin inhibitor on liver arginine esterase activity was also examined. At concentrations of STI up to 100 μg, there was little or no inhibition of arginine esterase activity in both normal liver and in liver from Cystic Fibrosis (Table 3). Thus, the arginine esterase of liver is quite different in its response to trypsin inhibitor from the enzyme found in plasma and saliva.

To identify the possible deficiency of one or more specific iso-enzymes, liver extracts were then subjected to isoelectric focusing as described. Eluates were assayed for the presence of cathepsin B_1, arginine esterase and for glycosidases. A single major peak of cathepsin B_1 was obtained (pI 4.9) in normal and CF liver (Figure 3). Smaller amounts of activity were found at pI values below this isoelectric point. A small discrete peak of activity was also seen at pI 5.1 in both normal and in CF liver. Arginine esterase was measured in the eluates with BAEE as substrate. Two partially resolved peaks of activity were noted. The first had a pI in the range of 5.6-5.7 while the second had a pI of 5.8-5.9. There was no difference in the activity profile of arginine esterase when assayed in the presence of 100 μg of soybean trypsin inhibitor. The recovery of cathepsin B_1 activity was 37-47 % for both normal and CF liver while arginine esterase recoveries were much better (67-75%). Glycosidases such as β-hexosaminidase, β-galactosidase and α-galactosaminidase were monitored as internal controls and were always normal.

DISCUSSION

The properties of cathepsin B_1 presented in this work (pH optimum, Km value, isoelectric point) confirm the findings of Barrett (2) and extend the data to include liver from Cystic Fibrosis patients. A previous report suggested the possibility that a carboxypeptidase or cathepsin B-like enzyme may be involved in CF (10,17). The present data seem to preclude a deficiency in Cathepsin B_1 in Cystic Fibrosis. Other lysosomal hydrolases such as α-mannosidase, α-fucosidase, β-hexosaminidase, β-galactosidase and sphingomyelinase gave normal or slightly elevated values with respect to total activity.

Several isoelectric focusing experiments on liver extracts and also on extracts of cultured skin fibroblasts have shown a normal isoenzyme pattern for these hydrolases with the possible exception of α-fucosidase (unpublished observations; also see reference 1).

Rao and Nadler have shown that arginine esterase of human plasma and saliva can be resolved into two separate peaks of activity by DEAE-cellulose chromatography (16). The major enzyme is inhibited by trypsin inhibitor while the minor one is not. In liver, with both BAEE and TAME the pH optimum is more basic than for the enzyme in plasma and the liver enzyme is not inhibited by soybean trypsin inhibitor. However, preliminary experiments on the plasma enzyme with TAME as substrate have confirmed the presence of enzymes which can be activated by chloroform or elagic acid. Thus, it seems that the arginine esterase in liver is different from the majority of the enzyme in human plasma and saliva. It may be similar to or the same as the minor component which has been shown to be unaffected in CF (15,16).

The major component of plasma arginine esterase is presumed to be derived from the pancreas and other exocrine tissues (18). It would thus be of interest to compare the levels of this enzyme in matched groups of CF patients, some of whom have marked pancreatic insufficiency and others who have normal or slightly altered pancreatic function. Such a study would show if the reduction in STI inhibitable arginine esterase activity observed in the studies of Rao and Nadler (14-17) was chiefly due to inadequate secretion of these enzymes by exocrine tissues.

In summary, we have examined Cathepsin B_1, arginine esterase and other lysosomal hydrolases in liver from control tissues and from patients with Cystic Fibrosis. We have not been able to detect any marked abnormalities in the properties or the isoenzyme distribution of any of these enzymes in Cystic Fibrosis liver. Since one would expect the genetic defect to be expressed in most if not all tissues, it is likely that the enzymes described here do not reflect the primary genetic defect in this disease.

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TABLE I

LYSOSOMAL HYDROLASES IN CONTROL AND OF LIVER +

Liver was homogenized and centrifuged as described. All numbers are the averages of duplicate samples analyzed as outlined in Materials and Methods.

		Cathepsins			Glycosidases				Sphingomyelinase
		A	B_1	D	$G_{M1}\beta gal$	β hex	α Mann	α Fuc	
NORMAL	1.	353	496	1597	22.5	5.56	4.41	113.1	6.8
	2.	400	649	931	11.7	2.12	1.50	30.1	10.0
GAUCHER		766	906	5308	28.3	10.46	2.84	18.5	28.3
G_{M1}-GANGLIOSIDOSIS		1399	2677	7727	0.1	20.36	30.33	163.0	65.0
CYSTIC FIBROSIS	1.	446	632	1156	14.5	2.49	2.14	61.7	10.3
	2.	888	1115	2824	38.8	3.59	1.56	63.4	28.8

+ All specific activities are reported in nanomoles substrate hydrolyzed or product released per hour per mg protein except for β-hexosaminidase where the units are μmoles product released/hour/mg protein.

TABLE 2

KINETIC CONSTANTS OF CATHEPSIN B_1 AND ARGININE ESTERASE IN LIVER +

		Cathepsin B_1				Arginine Esterase	
		+ Cysteine		- Cysteine			
		Km (mM)	V Max (nm/20 min)	Km (mM)	V Max (nm/20 min)	Km (mM)	V Max (OD/min)
NORMAL							
	1.	2.97	43.9	1.76	14.5	0.29	0.03
	2.	2.93	55.5	0.76	13.0	0.37	0.04
CYSTIC FIBROSIS							
	1.	3.39	55.9	2.76	15.5	0.35	0.04
	2.	2.47	55.9	0.77	7.5	0.45	0.02

+ The experimental data were analyzed by two way linear regression and the numbers presented were computed from the theoretical equation.

TABLE 3

ARGININE ESTERASE IN NORMAL AND CF LIVER +

		BAEE		TAME
		No STI	+ STI	No STI
NORMAL				
	1.	0.40	0.40	0.13
	2.	0.32	0.32	0.11
	3.	0.43	0.42	0.11
GAUCHER		0.47	0.49	-
G_{M1}-GANGLIOSIDOSIS		0.36	0.39	-
CYSTIC FIBROSIS				
	1.	0.20	0.20	0.09
	2.	0.20	0.19	0.09

+ Specific activity is expressed as Δ OD $_{253}$/min/mg protein for BAEE hydrolysis and as μmoles methanol released/h/mg protein for TAME hydrolysis. Soybean trypsin inhibitor (100 μg) was included in the incubation mixtures where indicated.

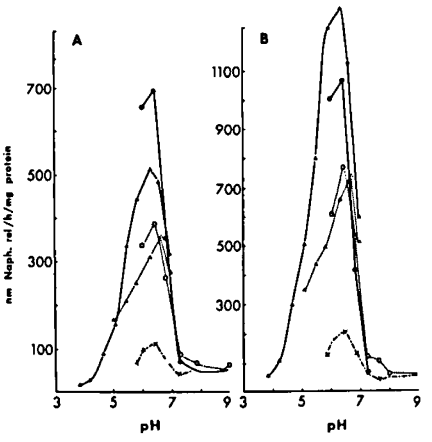

Figure 1

Cathepsin B_1 activity as a function of pH

Cathepsin B_1 activity was measured in normal (A) and CF liver (B) in the presence of 2.67 mM cysteine (closed symbols), in the absence of cysteine (open symbols) and in the presence of 10 mM hydroxymercuribenzoate (-x-). Citrate-phosphate (triangles) and phosphate (circles) buffers were used. All points are the averages of duplicate measurements. Incubations were carried out for 20 minutes as described according to Barrett (2).

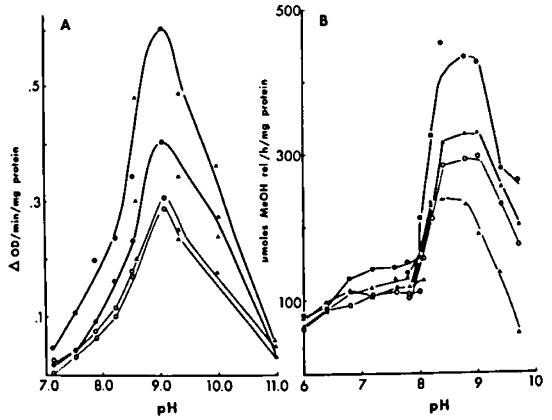

Figure 2

Arginine esterase activity as a function of pH

Arginine esterase activity was measured with BAEE (A) and TAME (B) substrates. In both sets of experiments two normal (solid symbols) and two CF livers (open symbols) were compared. In panel A, the buffers used were Tris-HCl (circles) and Glycine-NaOH (triangles) while in panel B, phosphate buffer was used from pH 6-8 and 2-amino-2-methyl-1: 3-propanediol-HCl buffer was employed from pH 7.8-9.7. All points are averages of duplicate measurements.

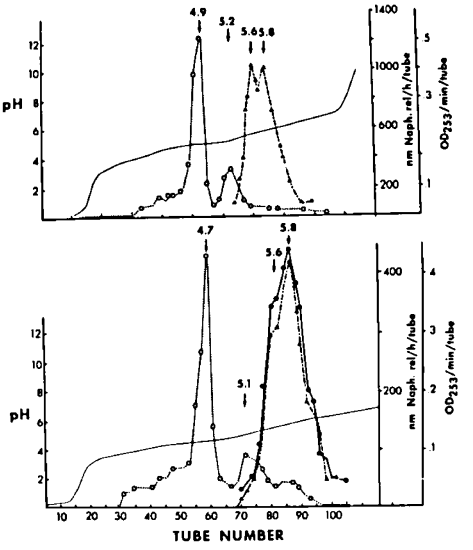

Figure 3

Isoelectric Focusing of Cathepsin B_1 and Arginine Esterase in human liver

Representative experiments of normal (lower panel) and CF liver (upper panel) are shown. Cathepsin B_1 (open circles) was resolved into one major peak of activity with minor peaks of enzyme spread throughout the gradient. Arginine Esterase (solid triangles) was partially resolved into two components in the CF liver but the resolution was less complete for the control (pI values 5.6 and 5.8). Soybean Trypsin Inhibitor (100 μg/ml) had no effect on the Arginine Esterase activity (solid circles). The pH (shown as a solid line) was measured in alternate fractions (0.8-1.0 ml).