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Liver pathology in a new congenital disorder of urea synthesis: N-acetylglutamate synthetase deficiency

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Summary. Detoxification through the urea cycle is the means by which mammalian organisms dispose of their excess ammonia. Within this cycle, N-acetylglutamate (NAG) is the most important cofactor for optimal enzyme activity. It is formed from acetyl CoA and glutamate through the action of N-acetylglutamate synthetase (NAGS). Recently, a congenital deficiency of NAGS has been reported. In this communication, we present results of structural investigations on liver tissue of the index patient with NAGS defect.

Light microscopy revealed small, eosinophilic inclusions in some of the hepatocytes. Electron microscopy showed vesicular SER and fibrillar material in expanded cisterns of the RER, presumably corresponding to the inclusions seen in light microscopy. Immunofluorescence of liver tissue uncovered a discrete distribution of intracellular albumin in the form of small deposits. We suggest that in NAGS deficiency, some secretory proteins might be incompletely processed due to the lack of a protease activator, NAG.

Key words: Urea cycle defects – N-acetylglutamate synthetase deficiency – Liver pathology

Introduction

Detoxification through the urea cycle is the essential means by which mammalian organisms dispose of their excess ammonia. The complex organization of ureagenesis is illustrated in Fig. 1. Inadequate function of the system will lead to hyperammonaemia and its sequelae. While hyperammonaemia in adults is most commonly caused by liver disease, it may result from a variety of inborn errors of metabolism in children. They comprise urea cycle enzyme deficiences, abnormalities of transport of urea cycle intermedi-

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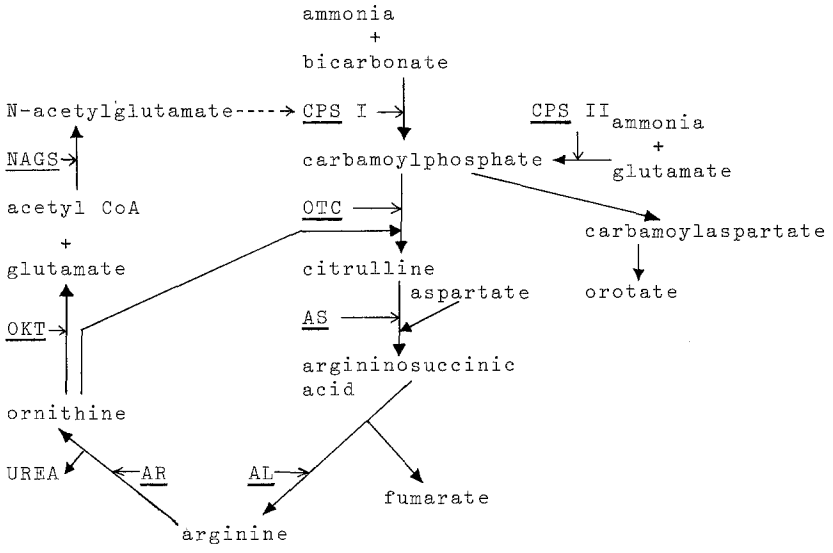

Fig. 1. The urea cycle. *CPS* I carbamoylphosphate synthetase I (mitochondrial); *CPS* II carbamoylphosphate synthetase II (cytosolic); *OTC* ornithine transcarbamylase (mitochondrial); *AS* argininosuccinate synthetase (cytosolic); *AL* argininosuccinate lyase (cytosolic); *AR* arginase (cytosolic); *NAGS* N-acetylglutamate synthetase (mitochondrial); *OKT* ornithine-ketoacid transaminase (mitochondrial). N-acetylglutamate activates *CPS* I (*arrow* with broken line)

ate metabolites, or organic acidaemias, including valproate therapy (for review, see Walser 1983). Heritable defects of urea synthesis may result in different patterns of metabolite accumulation or product deficiency apart from hyperammonaemia depending on the site of the block. Thus, the pathological consequences will vary among the disorders as a function of the specific cell and tissue distribution of a given enzyme and of the in vivo relevance of a metabolic block for each cell population. In order to further clarify the complex pathogenesis, there is a need for more detailed information on structural derangements in relation to functional sequelae of the defects detected so far. Ultrastructural changes have been reported for carbamoylphosphate synthetase (CPS) and ornithine transcarbamylase (OTC) deficiences (Hug et al. 1978; La Brecque et al. 1979; Shapiro et al. 1980; Zimmermann et al. 1981) and for citrullinaemia (Mihatsch et al. 1974).

Recently, Bachmann and coworkers (1981a, 1981b) have described the first case of hyperammonaemia due to congenital deficiency of N-acetylglutamate synthetase (NAGS). In the present communication we report results of structural investigations on liver tissue of this unique disorder and their possible interpretation.

Case report

The clinical data of the patient and the elucidation of the enzyme defect are given in detail elsewhere (Bachmann et al. 1981a, 1981b). In brief, the male patient was a full-term infant

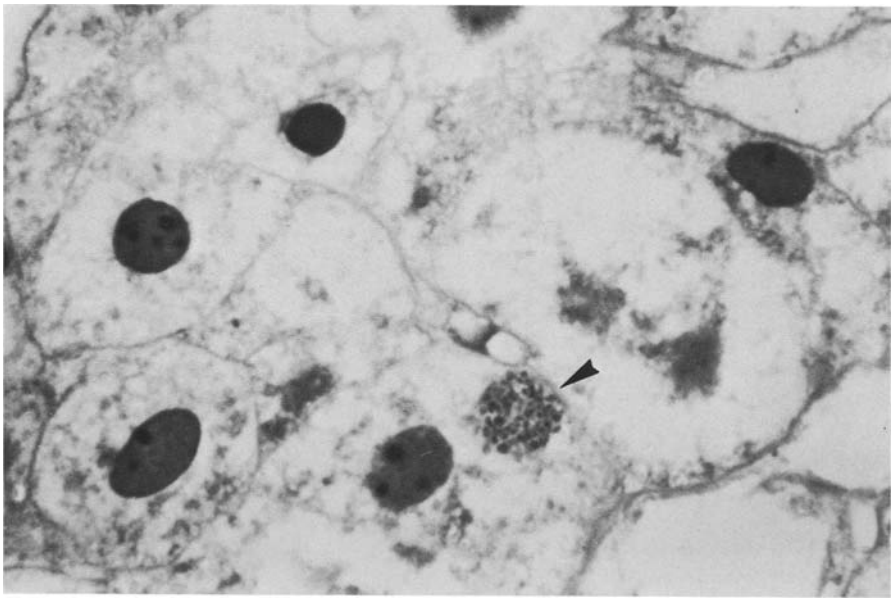

Fig. 2. Liver tissue, first biopsy. The hepatocytes exhibit a clear cytoplasm. Note the dense cytoplasmic inclusions (*arrow*; hematoxylin and eosin, ×400)

who developed hyperammonaemia on the third day of life. NAGS activity was undetectable in liver tissue. Two male siblings had died in the newborn period (at days 5 and 11, respectively); we have no specific biochemical data on these patients. The index patient was treated initially with sodium benzoate (the coenzyme A-ester of benzoate combines with glycine to form hippurate, which is efficiently excreted by the kidneys), with nitrogen restriction (nitrogen intake: 32 mM/kg/day), with arginine (2.64 mM/kg/day; total intake, the food included: 3 mM/kg/day; arginine is needed for the synthesis of proteins, creatine and ornithine) and later on with carbamoylglutamate (320 mg/kg/day; this compound is a congener of NAG, which is not catabolized by cytosolic acylase and will penetrate into mitochondria; Bachmann and Colombo 1982). The treatment resulted in a decrease of ammonia levels, and an increase of both the serum urea nitrogen level and the urinary excretion of orotic acid. The boy has now survived for $4^1/_2$ years but is however hypotonic and mentally retarded (DQ 64%).

Material and methods

Liver tissue samples were obtained through biopsy on two occasions. At the first biopsy, the patient was four days old and had been treated with sodium benzoate (227 mg/kg) for two days. The second liver biopsy was performed at the age of eight months while under treatment with arginine and carbamoylglutamate.

Liver tissue was either conventionally processed for light microscopy or was fixed with glutaraldehyde, postfixed in OsO_4, and further processed for electron microscopy. For the demonstration of specific intracellular proteins, sections of formaline-fixed tissue were incubated with FITC-labeled antisera containing specific antibodies directed against determinants of albumin, fibrinogen and alpha-1-antitrypsin (AAT). The preparations were examined in a Leitz Ortholux microscope (incident light Ploemopak, excitation at 450–490 nm, using water immersion objectives). Age-matched, normal human liver tissue, processed in parallel, served as a control.

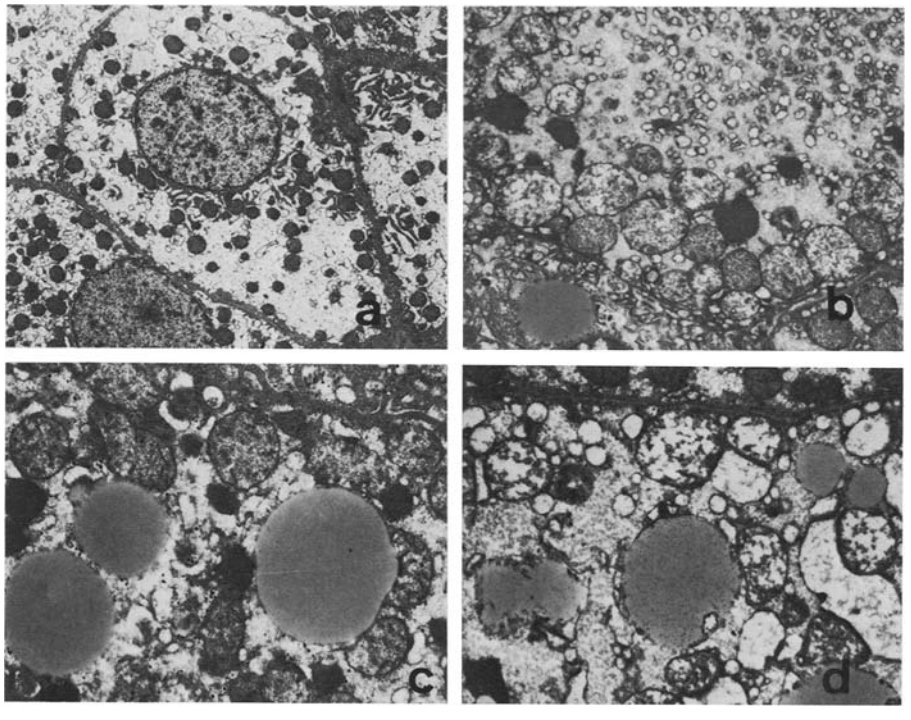

Fig. 3a–d. Electron micrographs of hepatocytes. **a** The cell in the center presents a clear cytoplasm reflecting the aspect noted at the light microscopic level (original magnification × 5,000, reduced). **b** At higher magnification a finely vesicular SER is seen (top half of the figure × 20,000, reduced). **c** Several mitochondria are seen with, apart from some autolytic change, normal structure. Note three lipid droplets (× 25,000, reduced). **d** The right half of the figure shows dilated cisterns of the RER with a flocculent material (× 25,000, reduced)

Results

The morphological features of the first liver tissue sample as revealed by light microscopy are shown in Fig. 2. The structure of the liver was preserved, and the hepatocytes presented a rather pale or clear cytoplasm. Portal tracts and sinusoids were normal. Cholestasis, fatty change, necrosis or cellular infiltrates were lacking. Some of the hepatocytes contained small groups of strongly eosinophilic, PAS-negative cytoplasmic inclusions, measuring up to 0.5 μm (Fig. 2, arrow). Electron microscopic examination of liver tissue obtained at the age of 4 days (Fig. 3) revealed hepatocytes with a prominent smooth endoplasmic reticulum (SER) combined with an abundance of glycogen particles. SER vesiculation and glycogen deposition are well in line with the finding of pale cytoplasm in light microscopy. Interestingly, the cisterns of the ribosome-coated profiles of the rough endoplasmic reticulum (RER) were dilated and deformed, and contained a finely granular or filamentous material of moderate electron density (Fig. 4). In some of the expanded cisterns, filamentous structures were associated with the inner surface of the RER membrane. Similar findings were obtained with liver

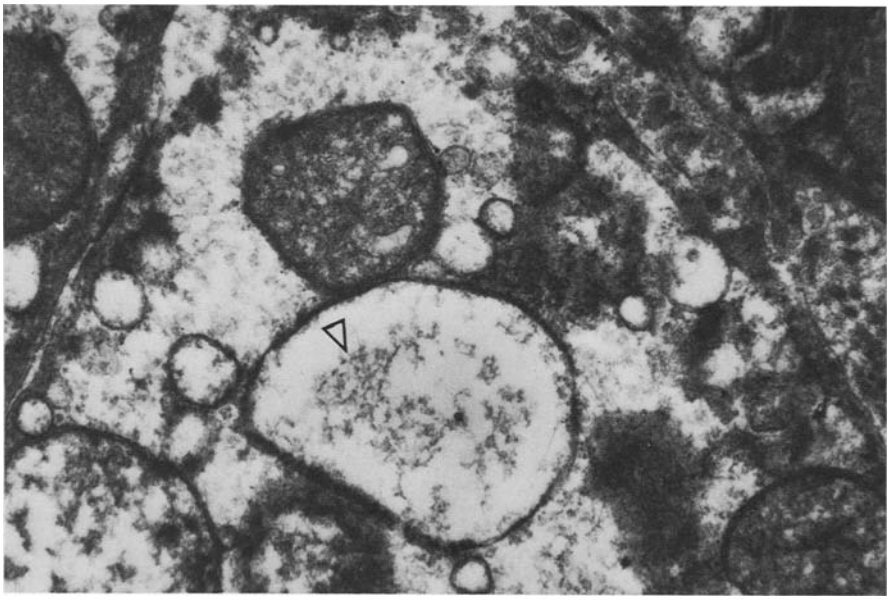

Fig. 4. Electron micrograph of a hepatocyte. The RER cistern shown in the center is dilated and contains moderately electron dense, filamentous material (*arrow*; × 25,000)

tissue taken at the age of 8 months (not shown). In both biopsies, relevant changes of mitochondria, or the formation of residual bodies, were not seen. In order to test if the material apparently accumulated within RER cisterns might correspond to a deposition of a specific secretory (export) protein within hepatocytes we analyzed liver sections for the presence and distribution of albumin, fibrinogen and AAT as typical representatives of liver-specific export proteins. As seen in Fig. 5a, positive immunofluorescent staining for albumin was evenly distributed in control hepatocytes. In contrast, the albumin distribution in the liver cells of the patient was characterized by rather small concentrated cytoplasmic deposits (Fig. 5b). In some of the cells, positive staining was noted at the cellular periphery. The reaction for fibrinogen was sparse and similar in both the patient's liver and the control material (not shown). AAT was not detected. As extensive search for any other source of the inclusions seen in light microscopy was negative, we assume that the alterations seen in EM and in immunofluorescence microscopy correspond to the eosinophilic structures observed in hepatocytes.

Discussion

N-acetylglutamate (NAG) is a critical activator of the first step of urea synthesis catalyzed by carbamoylphosphate synthetase I (CPS I). NAG acts as an allosteric effector through binding to the enzyme and maintaining

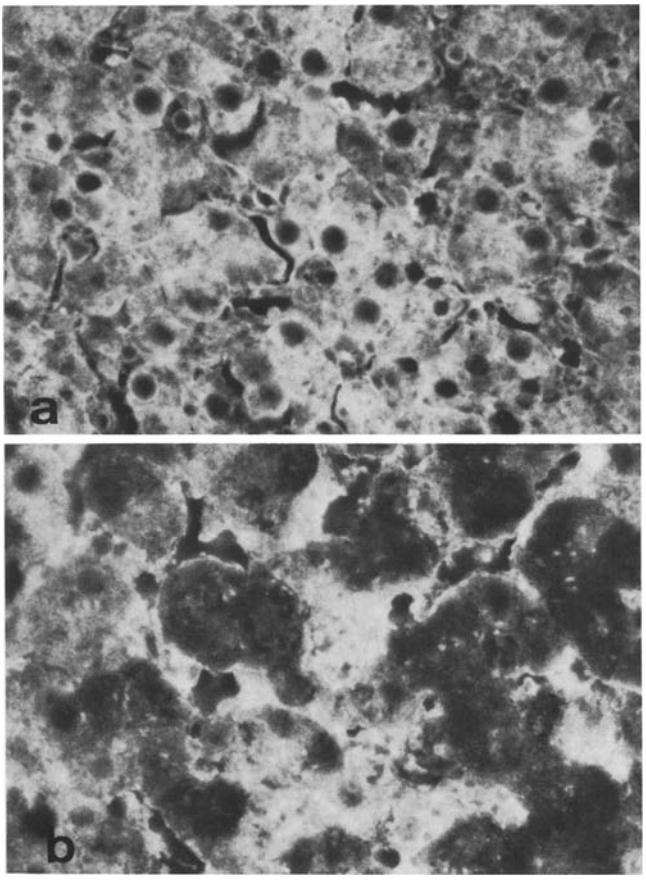

Fig. 5a, b. Sections of liver tissue incubated with FITC-labeled antibodies directed against human albumin. **a** Age-matched control. Hepatocytes are seen to exhibit a diffuse reaction in their cytoplasm, sparing the nuclei. **b** Liver tissue in NAGS deficiency. Note that, in contrast to **a**), fluorescence shows a discrete distribution. In some hepatocytes it is visible only in the form of cytoplasmic dots or of thin bands along the cell periphery

it in its active form. It thus stimulates the synthesis of carbamoylphosphate from ammonium ions and bicarbonate within hepatic mitochondria. NAG is formed from acetyl CoA and glutamate through the action of the mitochondrial matrix enzyme, N-acetylglutamate synthetase (NAGS), which is stimulated by arginine. A lack of NAG leads to a severe reduction of CPS I activity (less than 5% of normal) and thus mimics primary deficiency of CPS I.

In liver tissue obtained from the index patient with NAGS deficiency, no relevant hepatic changes could be found with light microscopic examination, except for eosinophilic inclusions in the cytoplasm of hepatocytes. Hence, the NAGS defect is similar to other heritable disorders of the urea cycle, where light microscopic analyses alone have shown that alterations

of the liver, if present at all, are largely non-specific (no detectable anomaly: Levin et al. 1969; Saudubray et al. 1973; Campbell et al. 1973; Farriaux et al. 1974; Goldstein et al. 1974; Ricciuti et al. 1976; fatty change: Corbeel et al. 1969; Hommes et al. 1969; Hopkins et al. 1969; Freeman et al. 1970; Matsuda et al. 1971; Vidailhet et al. 1971; Mihatsch et al. 1974; La Brecque et al. 1979; cholestasis: Mihatsch et al. 1974; Leibowitz et al. 1978). Abundance of glycogen in hepatocytes, as seen in the present case, is probably a consequence of the high intravenous glucose supply given to these patients, and has also been reported for other defects (Hopkins et al. 1969; Bruton et al. 1970; Hug et al. 1978), while a decrease of hepatocytic glycogen has been observed in OTC deficiency (Sunshine et al. 1972). Fatty change and/or cholestasis, not found in NAGS deficiency, usually indicate a nonspecific liver damage in phases of compromised hepatocytic function, as occasionally seen in congenital hyperammonaemia. We would like to stress that, at least in the biopsy material obtained up to now, an increase of hepatic fibrous tissue has not been observed in the NAGS defect. In contrast other heritable defects of the urea cycle appear to be prone to develop hepatic septal fibrosis: variable degrees of liver fibrosis have been reported for OTC deficiency (Hopkins et al. 1969; La Brecque et al. 1979; Shapiro et al. 1980), in part associated with piecemeal necrosis (La Brecque et al. 1979), and in CPS deficiency (personal observation). We also recently studied a patient with argininosuccinic aciduria where severe liver fibrosis was associated with incipient cirrhosis. As arginine deficiency might represent one factor in the pathogenesis of fibrous liver change (Bachmann 1984), it appears crucial to supplement carefully this essential amino acid (or citrulline) in patients with heritable defects of the urea cycle (except for arginase deficiency), as we did with our NAGS-deficient patient.

A peculiar finding in NAGS deficiency is the presence of eosinophilic inclusions in the cytoplasm of hepatocytes. We have evidence that these bodies correspond, on an ultrastructural level, to dilated cisterns of the RER containing a granular and fibrillary material. Viral products or intermediate filament clusters as seen with Mallory bodies were not observed. The strongly eosinophilic character of the inclusions points to their protein nature. A glycoprotein is probably not involved since the PAS reaction was negative. In their morphology the inclusions are reminiscent of deposits found in some congenital disorders of hepatocytic protein turnover. In alpha$_1$-antitrypsin deficiency (AAT-D), protein inclusions in hepatocytes are well known; they are PAS-positive (Berg and Eriksson 1972; Eriksson and Larsson 1975). Sharp (1971) was the first to demonstrate the presence of globules in the hepatocytes of children with homozygous (ZZ) AAT-D, and the inclusions have since been identified as corresponding to the Pi^z variant, containing inactive AAT within cisterns of the RER (Reintoft 1979). A block in release of incomplete AAT is discussed, in that a loss of sialic acid residues due to variations of charged amino acids in variant AAT may account for the putative export defect (Yoshida et al. 1976; Lieberman 1980). Another phenotype, the $Pi^{Mduarte}$ variant, may be associated with liver cell globules (Lieberman et al. 1976). They can, however, also be found

with normal PiMM phenotypes and normal levels of AAT (Fisher et al. 1976). These so-called non-AAT globules are thought to result from a peculiar type of liver cell necrosis (Kern et al. 1969). AAT could not be detected in our case. The tests for fibrinogen revealed no inclusions, what is of interest with regard to the finding of deposits in congenital hypofibrinogenaemia (Pfeifer et al. 1981).

The observation of a discrete distribution of albumin reactivity in hepatocytes of our case is, however, important in the light of the fact that alpha-fetoprotein (AFP) the fetal analog of albumin, is found together with AAT within globules of hepatoma cells in the absence of AAT-D (Palmer et al. 1980), and in the hyaline inclusions of endodermal sinus tumours (Palmer and Wolfe 1978). AFP appears to be stored in the cisterns of the RER (Gonzales-Crussi and Roth 1976) and the phenomenon is interpreted to mean that a fetal export protein gene is re-expressed in these neoplastic cell populations. In contrast, the accumulation of albumin apparently present in our case calls for a different interpretation. It may either represent a derangement of intracellular protein processing, be related to therapy, or may correspond to a phenomenon independent from NAGS deficiency. Benzoate, which conjugates with glycine to yield hippurate, is currently regarded as nontoxic (Batshaw et al. 1982). However, the process consumes ATP and, if glycine is low, benzoyl CoA will accumulate and might impair gluconeogenesis and lipogenesis (McCune et al. 1982). We have no evidence that benzoate could be a relevant factor in the pathogenesis of the changes observed in our patient as it was no longer given when the second biopsy was performed. However, carbamoylglutamate, a second therapeutic component, had not yet been applied at the time of the first biopsy.

A deficiency of NAG itself (or NAGS deficiency) could theoretically result in a disorder of the intracellular processing of secretory proteins. Export proteins such as albumin are synthesized on polyribosomes, translocated through RER membranes and then post-translationally modified via limited proteolytic splitting. Trypsin- and carboxypeptidase B-like enzymes are involved (Jackson and Blobel 1977). The transformation of pre-proproteins into the smaller molecular species is a crucial step within the mechanisms leading to efficient protein export. NAG is considered to be a physiological activator of proteolytic enzymes at neutral pH (Grisolia 1976). We theorize that a lack of NAG may result in a suboptimal activation of cellular endopeptidases and thus in the accumulation of incompletely processed secretory proteins.

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