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Developmental Studies on Glucosamine Metabolism (39455)

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Glucosamine and its derivatives are unique and obligatory structural components of many biologically important macromolecules, including membrane glycoproteins and glycolipids, plasma glycoproteins, and glycosaminoglycans. During postnatal development, from birth to the early adult stage, a great deal of rapid growth and maturation occurs. One might expect that this would be reflected in an increased rate of synthesis of various hexosamine-containing macromolecules since these are components of all cells, tissues, and biological secretions. This increased synthetic rate should be reflected, in turn, in the rate of synthesis of glucosamine and its derivatives.

The present paper reports the results of assays of the activities of two key hepatic enzymes involved in hexosamine synthesis and conversion during postnatal development. In addition, the hepatic concentrations of two glucosamine metabolites involved in the feedback regulation of these enzymes were measured. Specifically, the enzymes examined were L-glutamine:D-fructose-6-phosphate aminotransferase (AT) and UDP-*N*-acetyl-glucosamine 2′-epimerase (EP). The metabolites measured were UDP-*N*-acetylglucosamine (UDP-GlcNAc) and CMP-*N*-acetylneuraminic acid (CMP-NANA).

Materials and methods. Animals. Male, Wistar-King A, rats bred, born, and raised in this laboratory were maintained on standard laboratory chow and housed until used in environmentally controlled animal quarters.

Chemicals. UDP-*N*-acetylglucosamine, *N*-acetylneuraminic acid, and fructose-6-phosphate were purchased from Sigma. All other chemicals were of the highest quality available from commercial sources.

Enzyme preparation. Rats were anesthetized with ether and the livers were exposed through an abdominal incision. Blood was withdrawn from the abdominal aorta for seromucoid determinations. The livers were perfused via the portal vein with an ice-cold solution of 0.1 *M* phosphate buffer, pH 7.5; then were removed, blotted, weighed, and homogenized in 2 vol of ice-cold 0.1 *M* phosphate buffer–1 m*M* EDTA, pH 7.5, for 1 min using a Polytron homogenizer Model 10ST operated at 10,000 rpm. The homogenate was centrifuged for 10 min at 20,000*g* and the postmitochondrial supernatant was removed and centrifuged for 60 min at 105,000*g*. The clear, middle portion of this supernatant served as the source of the enzyme activities.

Assay of L-glutamine-D-fructose-6-phosphate aminotransferase. L-glutamine:D-fructose-6-phosphate aminotransferase (AT) was assayed as previously described (1) according to the method of Kornfeld (2). Each reaction mixture contained 10 m*M* fructose-6-phosphate, 10 m*M* glutamine, 1 m*M* EDTA, 40 m*M* sodium phosphate buffer, pH 7.5, and 0.1 ml enzyme extract in a total volume of 0.5 ml. After incubation at 37° for 60 min, the reaction was terminated by heating in a boiling-water bath for 1 min. The protein precipitate was removed by centrifugation and a 0.3-ml portion of the supernatant was assayed for glucosamine-6-phosphate by the method of Levvy and McAllan (3).

Assay of UDP-N-acetylglucosamine 2′-epimerase (EP). The procedure used was based upon the measurement of *N*-acetylmannosamine produced in the reaction mixture using a modified Morgan-Elson procedure as described by Spivak and Roseman (4). The assay mixture contained 2 m*M* UDP-GlcNAc, 200 m*M* Tris-HCl buffer, pH 7.5, 80 m*M* $MgSO_4$, and 0.1 ml enzyme extract in a total volume of 0.25 ml. Incubation was carried out at 37° for 20 min and the reaction was terminated by heating in a boiling-water bath for 2 min. The protein

precipitate was removed by centrifugation and a 0.1-ml aliquot of the supernatant was placed in a test tube along with approximately 80–100 mg of Dowex-2 acetate (20 mesh). Three-tenths milliliter of water was added to increas the volume. The tubes were mixed vigorously and the reaction mixture was allowed to react with the resin for about 5 min. The resin was removed by centrifugation and 0.2 ml of the supernatant was used for the estimation of N-acetylmannosamine.

Protein assay. The protein content of the 105,000*g* supernatant was determined by a modified biuret method according to Wolfson *et al.* (5) using crystalline bovine albumin as a standard. Enzyme specific activities were expressed as nanomoles of glucosamine-6-phosphate formed per milligram of protein per hour for AT and as nanomoles of *N*-acetylmannosamine formed per milligram of protein per hour for EP.

UDP N-acetylglucosamine assays. The concentrations of hepatic UDP-GlcNAc were determined as previously described (1) by a modification of the procedure of Bates and Handschumacher (6). One volume of 50% w/v trichloroacetic acid (TCA) was added to 9 vol of 105,000*g* supernatant. After removing acid-insoluble material by centrifugation, the supernatant was heated 10 min in a boiling-water bath to hydrolyze UDP-*N*-acetylhexosamines. The solution was centrifuged again to remove heat-coagulated material and 0.5 ml of this supernatant was assayed for hexosamine content by a modification of the Elson–Morgan reaction as described by Neuhaus and Letzring (7). A standard curve was prepared using glucosamine hydrochloride. The UDP-GlcNAc pool sizes were expressed as nanomoles of UDP-GlcNAc per gram of liver.

Assay of CMP-N-acetylneuraminic acid. The hepatic concentrations of CMP-NANA were determined on the same TCA and heat-treated supernatants used to assay UDP-GlcNAc pool sizes. The method used for CMP-NANA measurements was that of Jourdian *et al.* (8). Five-tenths milliliter of the TCA supernatant was added to 0.1 ml of 0.04 *M* periodic acid solution, mixed and allowed to stand in ice-cold water for 20 min. After the addition of 1.25 ml of resorcinol reagent, the solution was mixed, placed in an ice bath for 5 min and heated at 100° for 15 min. The reaction mixture was cooled in tap water, 1.25 ml of tertiary butyl alcohol was added and the mixture agitated vigorously. The tubes were placed in a 37° water bath for 3 min and cooled to room temperature, and the absorbance was measured at 630 nm.

Assay of the seromucoid fraction. The seromucoid fraction was separated from serum by the method of Winzler (9) and was expressed in terms of its protein concentration as assayed by the method of Lowry *et al.* (10).

Results. L-glutamine: D-fructose-6-phosphate aminotransferase (AT). The changes in AT activity following birth are shown in Fig. 1. The average specific activity of the enzyme for adult animals was 47.7 ± 1.3 nmoles/mg of protein/hr. The enzyme activity at 24 hr after birth was one-third of the adult level (17.9 ± 1.1 nmoles/mg/hr). The AT activity continued to increase until about 14 days after birth, at which point it reached 1.7 times the adult value. The peak value was followed by a gradual decrease over the next 2 weeks, gradually returning to the adult level. The specific activity of the enzyme in older adult animals tended to be slightly lower when compared to that of younger adult animals.

UDP-N-acetylglucosamine 2'-epimerase (EP) activity. As can be seen in Fig. 1 the

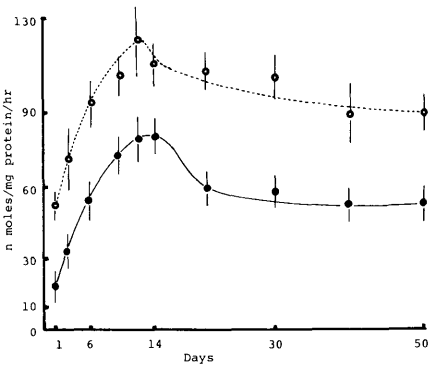

FIG. 1. Changes in specific activities of AT and EP during development. Values are presented as means ± SD. Each point represents the mean of four to six animals. ●——● L-glutamine:D-fructose-6-P aminotransferase (AT). O-----O UDP-GlcNAc 2' epimerase (EP).

developmental pattern of EP activity was quite similar to that of AT. The average specific activity of the EP for adult animals was 75.0 ± 6.3 nmoles/mg of protein/hr. The enzyme activity at 24 hr after birth was about half the adult value (39.3 ± 4.2 nmoles/mg/hr) and continued to increase to a maximum at about 14 days, at which point it reached 1.5 times the adult level. This was followed by a gradual decrease over the next 2 weeks to adult values.

UDP-N-acetylglucosamine concentration. The developmental changes occurring in the UDP-GlcNAc pool size are shown in Fig. 2. The UDP-GlcNAc pool size in adult livers averaged 436 ± 38 nmoles/g of liver in these experiments. Very high concentrations (963 ± 82 nmoles/g), approximately two times the adult value, were measured at 24 hr after birth. These values then continued to decline slowly over the next 20 days and after that decreased somewhat more sharply toward the adult level.

CMP-NANA concentrations. The developmental changes occurring in hepatic CMP-NANA concentration are shown in Fig. 3. As compared to UDP-GlcNAc, less dramatic changes were observed in the CMP-NANA pool with development. CMP-NANA maintained a fairly constant concentration until about 20 days after birth, decreased sharply between 20 and 30 days and then plateaued for the next 20 days. The CMP-NANA concentration of adult livers averaged 157 ± 13 nmoles/g liver in these experiments.

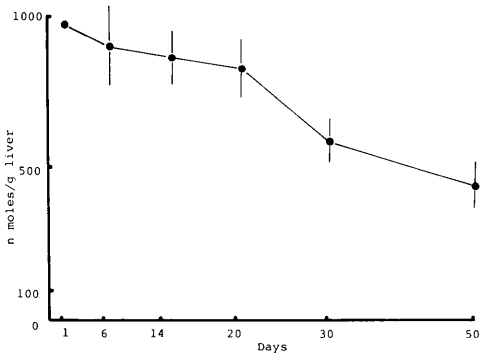

FIG. 2. Changes in hepatic UDP-GlcNAc concentration during development. Assays were carried out on the same 105,000*g* supernatant used to assay the enzyme activities presented in Fig. 1. Values are presented as means ± SD. Each point represents the mean of four to six animals.

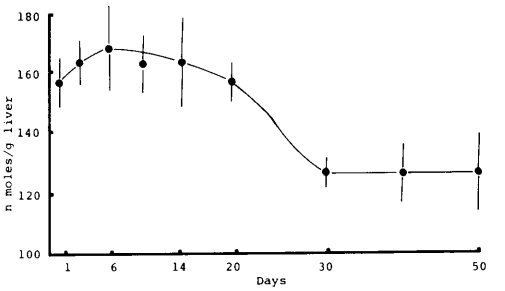

FIG. 3. Changes in hepatic CMP-NANA concentration during development. Assays were carried out on the same 105,000*g* supernatant used to assay the enzyme activities presented in Fig. 1. Values are presented as means ± SD. Each point represents the mean of four to six animals.

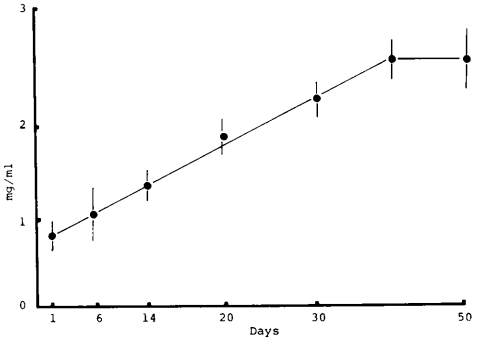

FIG. 4. Changes in serum seromucoid concentration during development. Blood was obtained from the abdominal aorta, and the seromucoid fractions were prepared by the method of Winzler (9). Values are presented as means ± SD. Each point represents the mean of four to six animals.

Seromucoid concentrations. The changes in seromucoid concentration with development are shown in Fig. 4. The average concentration of the seromucoid fraction for adult animals was 2775 ± 86 μg/ml of serum. Much lower concentrations, about one-third of the adult value, were measured at 24 hr after birth. The seromucoid concentration then continued to increase linearly with development to about the 30th day, after which the rate of increase declined to the adult level 50 days after birth.

Discussion. The general pattern of change observed in AT and EP specific activities agreed quite well with that reported by Kikuchi *et al.* (11), with the exception that they

observed a greater rate and extent of increase in the enzyme specific activities. Some differences between our observations and those of Kikuchi *et al.* (11) do exist, however, even though they may be of relatively minor importance overall. First, we observed lower AT specific activities in 1-day-old rats, our earliest observation time, than in adult rats. Kikuchi *et al.* (11), however, observed the opposite. Second, they reported that peak AT specific activity occurred at about 5–10 days while we observed the peak to occur at about 12–14 days. The pattern of EP development is almost identical in the two studies.

It is probable that the differences observed between the two studies are not significant, although it should be noted that our enzyme assays were carried out on 105,000*g* supernatant fractions, while those of Kikuchi *et al.* (11) were performed on 0–40% $(NH_4)_2SO_4$ fractions for EP and 40–60% $(NH_4)_2SO_4$ fractions for AT. It is possible that the presence of activators or inhibitors could influence assays of the enzymes when the crude 105,000*g* fractions are used even though they would be greatly diluted out during the assay procedure. Ammonium sulfate fractionation should remove these factors but then one wonders what effects the fractionation procedures may have on the enzyme activities. It is a matter of conjecture as to which method yields the more accurate perspective.

UDP-GlcNAc, the major feedback inhibitor of AT, exhibited high hepatic concentrations at birth which then decreased with development to adult levels at about 50 days. Since the steady-state concentration of any metabolite is the result of a balance between synthesis and removal, it is interesting to note that the pattern of hepatic UDP-GlcNAc concentration with development is almost the reciprocal of that for the seromucoid fraction. Both change continuously for the 35–40 days and then level off simultaneously at the adult level. A similar reciprocal relationship between the pattern of development for the seromucoid fraction and the hepatic concentration of CMP-NANA was also observed during this time period. If one assumes that the major fate of the hepatic UDP-GlcNAc and CMP-NANA is to be incorporated into the glycoproteins of the seromucoid fraction, then the instantaneous concentration of these nucleotide sugars would be a function of the rate at which they are being synthesized and transferred to the glycoproteins of the blood plasma. There is, of course, some influence of cell growth and proliferation, but quantitatively this would be minor compared to blood glycoprotein production and secretion.

The adult levels found for CMP-NANA concentrations obtained in these experiments agree well with the values reported by Harms *et al.* (12) for normal liver. Although the method employed actually measured *free* NANA, previous work has shown that almost all of the NANA found in 105,000*g* supernatants of liver tissue exists as CMP-NANA. Liver supernatants not subjected to hydrolysis show only traces of free NANA.

It is difficult to explain the increase in the older adult animals after the seromucoid levels have come to equilibrium, but may reflect developmental changes in sialyltransferase activity.

Both UDP-GlcNAc and CMP-NANA are known to be feedback inhibitors of the initial enzymes in their respective pathways (13). What effect the changes in the hepatic concentrations of these compounds has on the specific activities of these enzymes is difficult to evaluate, since it is clear that the feedback inhibition exerted can be very complex. Winterburn and Phelps (14) and Miyagi and Tsuiki (15) for example, have shown that the control of AT is not regulated by fluctuation in the UDP-GlcNAc concentrations but rather is due to alterations in the AT:UDP-GlcNAc binding constant evoked by the secondary effectors UTP, G-6-P, and AMP. Similar complex relationship may exist between EP and CMP-NANA. Further investigations involving measurements of the intracellular concentrations of such secondary effectors with development must be carried out before the relationship between enzyme activity and amino sugar nucleotides can be clarified.

Summary. Changes in hepatic hexosamine metabolism and serum seromucoid concentrations during postnatal development were investigated in the rat. A rela-

tively low activity of hepatic L-glutamine: D-fructose-6-phosphate aminotransferase (AT) was observed within 24 hr of birth. This rapidly increased to a maximum at about 2 weeks of age, followed by a decline to adult levels after another 2 weeks. The developmental pattern of hepatic UDP-*N*-acetylglucosamine 2′-epimerase (EP) closely resembled that of AT. It was relatively low in the newborn, increased to a maximum at about 2 weeks and then declined to adult values after another 2 weeks. The serum seromucoid concentration was low in new born rats and then increased gradually with development to adult levels at about 50 days.

The hepatic concentrations of UDP-GlcNAc and CMP-NANA both were relatively high at 24 hr after birth compared to values observed at 50 days. The developmental pattern observed for both over this time period was quite similar and was almost the reciprocal to that for the seromucoid fraction. After 50 days the concentration of UDP-GlcNAc remained constant at adult levels as did that of the seromucoid fraction, but the concentration of CMP-NANA showed a marked increase.

It is suggested that the hepatic concentrations of these amino-sugar nucleotides during development primarily reflect the demand for the hepatic synthesis and secretion of plasma glycoproteins as reflected in the seromucoid fraction.

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