PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 334–339 (1985)

Proteochondroitin Sulfate Is the Main Proteoglycan Synthesized in Fetal Hepatocytes (42185)

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Abstract. The synthesis of total and specific types of glycosaminoglycans (GAG) with emphasis on proteochondroitin sulfate (PCS) was studied in late embryonic and early postnatal liver parenchymal cells. In contrast to adult hepatocytes, which synthesize almost exclusively proteoheparan sulfate (PHS), PCS proved to be the major type of GAG synthesized in fetal hepatocytes (more than 60% of total GAG) whereas PHS contributes less than 40% of total GAG synthesis. Starting immediately after birth PCS synthesis in hepatocytes declines progressively, at the 6th postnatal day PCS formation is one-fifteenth of that measured in embryonic liver cells. Adult levels are reached around the 10th postnatal day. A significant portion of plasma membrane-associated proteoglycans in fetal hepatocytes is represented by PCS, its fraction declines in early postnatal life. Between the synthesis rate of PCS and [^{3}H]thymidine incorporation into DNA exists a strong positive statistic correlation ($r = 0.949$). In conclusion, fetal hepatocytes have a completely different profile of GAG synthesis characterized by preponderant production of PCS. This ability is lost early after birth but might be regained in hepatocellular carcinoma cells and parenchymal cells in chronically injured liver tissue developing fibrosis.

In normal liver proteochondroitin sulfate (PCS) contributes about 10 to 15% of total liver glycosaminoglycans (GAG), the predominant type of GAG is proteoheparan sulfate (PHS) amounting to about 65% of total GAG (1–5). Two pathologic conditions of the liver are known in which the fraction of PCS increases considerably, that are both experimental and human liver fibrosis (1–5) and hepatocellular carcinoma (3, 4), respectively. A comparable elevation of PCS is found in developing liver (6–8). The mechanism of accumulation and the possible cellular source of PCS in fetal liver and in mature pathologic liver are not known. Neither hepatocytes nor Kupffer cells from adult normal liver were shown to synthesize PCS, the former cell type produces exclusively PHS (9, 10), the latter cells do not synthesize significant amounts of GAG at all (11). Nonetheless, hepatocytes might be a candidate cell type for the production of PCS if it is shown that the profile of hepatocellular GAG synthesis is significantly changed under these conditions. In a first attempt to follow this hypothesis we studied in the present report the rate of synthesis of total and specific types of GAG, in particular that of PCS and PHS, in isolated late embryonic and early postnatal parenchymal liver cells in comparison to that of mature hepatocytes and analyzed the distribution of the main types of GAG in the liver cell membrane. In addition the synthesis rate of GAG was tried to relate to the changes of DNA synthesis in developing liver tissue. The results point to the capacity of fetal hepatocytes to synthesize significant amounts of PCS and show that this ability is rapidly lost during the first 10 days of postnatal life.

Materials and Methods. *Animals.* Male Sprague–Dawley rats (Lippische Versuchstieranstalt, Extertal, Germany) were used. Rat embryos were prepared between the 19th and 22nd day of gestation.

Materials. Na [^{35}S]sulfate (500–600 mCi/mmole) and [^{3}H]thymidine (6.7 mCi/mmole) were obtained from New England Nuclear Corporation, Boston. DNAse and papain (EC 3.4.22.2) were from Boehringer-Mannheim, GmbH, Germany; chondroitin AC- (EC 4.2.2.5) and ABC-lyase (EC 4.2.2.4) from Seikagaku Kogyo Company, Tokyo, Japan; collagenase (EC 3.4.24.3, from Clostridium histolyticum, type I) from Sigma Chemical Company, Munich, Germany. Dulbecco's modification of Eagle's medium was purchased from Flow Laboratories, Bonn, Germany.

0037-9727/85 $1.50

Isolation and incubation of hepatocytes from adult liver. In principle the procedure of Seglen was followed as it is described elsewhere (11, 12). In the final parenchymal cell suspension the number of cells, the fraction of single parenchymal cells, and the cell viability by exclusion of trypan blue in the final concentration of 0.25% (w/v) were determined. The concentration of parenchymal cells in Dulbecco's modification of Eagle's medium was adjusted to 1×10^7 cells, which were incubated for 2 h at 37°C and 90 oscillations/min in 3 ml of Dulbecco's modification of Eagle's medium (pH 7.4) under 95% O_2–5% CO_2 with 25 μCi of [^{35}S]sulfate. At the end of incubation the cells were centrifuged, rinsed with ice-cold buffered saline, and transferred immediately into about 10 ml of cold acetone.

Isolation and incubation of embryonic and early postnatal liver parenchymal cells. The method of Devirgiliis *et al.* (13) was used for the preparation of embryonic and early postnatal parenchymal cells. The livers from embryonic and decapitated newborn rats were quickly excised and sliced with a razor blade in ice-cold Hanks' balanced salt solution containing 0.6 mmole/liter EGTA and 20 g/liter bovine serum albumin (medium A). The slices were freed of blood and about 1 g of liver tissue was incubated in a shaking water bath for 10 min at 39°C in 7 ml medium A. The supernatant was discarded, the tissue slices were incubated three times each for 15 min at 39°C with 7 ml of Hanks' balanced salt solution containing 0.005 mole/liter $CaCl_2$, 1.5 g/liter collagenase, and 0.05 g/liter DNAse. After each incubation the cells were filtered through nylon gauze (diameter of the pores 100 μm) and diluted 1:1 (v/v) with Dulbecco's modification of Eagle's medium. The cells were pelleted by centrifugation for 2 min at 40*g* and this cycle was repeated one time. The cells were examined microscopically as described for adult liver cells.

The procedure results in an embryonic liver cell suspension containing besides hepatocytes mainly erythroblasts. The incubation of the mixed cell suspension with [^{35}S]sulfate was performed as described above for adult liver cells. To separate hepatocytes from the nonparenchymal cells the adhesion of fetal hepatocytes to serum-coated plastic petri dishes was utilized. Two milliliters of Dulbecco's modified Eagle's medium containing 3% fetal calf serum were filled in petri dishes and stored for 5 hr at 4°C. Immediately before use the medium was sucked off and the dish was warmed up to 37°C. Cells (10^7) of the mixed suspension were poured in the dish in which they were incubated for 30 min at 37°C under 95% O_2–5% CO_2. Thereafter nonattached cells (mainly erythroblasts) and medium were sucked off, the plate was washed with 2 ml of ice-cold phosphate-buffered saline and the remaining cells (90–95% hepatocytes) were subjected to papain proteolysis for the isolation of glycosaminoglycans as described below.

Preparation and incubation of liver slices. After decapitation of the mother rat, embryos were quickly removed, their livers excised and washed in ice-cold phosphate-buffered saline. In principal the procedure described previously in detail for the preparation of liver slices was applied (14, 15). The conditions of incubations are very similar to those described for liver cells.

Determination of the incorporation of [^{35}S]sulfate into total and specific glycosaminoglycans. Cells were repeatedly washed (defatted) in cold acetone, chloroform/methanol (2/1, v/v), and ethanol/ethylether (3/1, v/v) after which the material was dried for 24 hr at 60°C (15). Thereafter it was homogenized in papain buffer (sodium acetate, 0.1 mole/liter, pH 6.2; sodium–EDTA, 0.05 mole/liter; cysteinium chloride, 0.005 mole/liter). An aliquot was taken for the determination of protein using bovine serum albumin as a standard (16). The homogenate was proteolyzed for 48 hr at 60°C by two consecutive additions of papain, each 12 U/ml. The proteolyzed, trichloroacetic acid soluble and neutralized supernatant was dialyzed overnight at 4°C against 100 vol of 0.03 mole/liter NaCl. Labeled total glycosaminoglycans together with added unlabeled glycosaminoglycans as carrier were precipitated with cetylpyridinium chloride as described elsewhere (14). The radioactivity of isolated glycosaminoglycans was measured and referred to the protein content.

To specify individual GAG types, total glycosaminoglycans were subjected to enzymatic degradation with chondroitin AC and ABC lyases to yield the incorporation of [^{35}S]sulfate into chondroitin 4,6-sulfate and dermatan sulfate, respectively, and to degradation with

nitrous acid to measure heparan [^{35}S]sulfate (14, 15). Synthesis of specific glycosaminoglycans is expressed either in absolute terms (dpm/mg protein) or relatively as fraction of total labeled glycosaminoglycans.

Determination of DNA synthesis. The incorporation of [^{3}H]thymidine into DNA was measured in liver slices (about 100 mg wet wt) and isolated liver cells exposed for 1 hr at 37°C to 2 μCi [^{3}H]thymidine in 3 ml Dulbecco's modification of Eagle's medium (17). After incubation, cells and slices, respectively, were washed in ice-cold phosphate-buffered saline and boiled for 5 min in water. The material was homogenized in water from which an aliquot was used for the determination of total protein (16), the remaining portion was precipitated with ice-cold 10% (w/v) trichloroacetic acid. After centrifugation (15 min, 3000g, 4°C) the sediment was washed twice in cold trichloroacetic acid and finally dissolved in 0.05 mole/liter NaOH for counting of radioactivity. The incorporated radioactivity is referred to the protein content of liver slices and cells, respectively.

Preparation of the plasma membranes. Cell membranes were isolated from whole rat liver and liver slices, respectively, labeled before with [^{35}S]sulfate. The preparation was performed by a modification (18) of previously described methods (19, 20). In brief, the liver was perfused with ice-cold 0.154 mole/liter NaCl containing 0.5 mole/liter $CaCl_2$, minced, and homogenized in an all-glass Dounce homogenizer. The homogenate was filtered through sterile gauze and centrifuged (15 min, 1500g, 0–2°C) four times. The final sediment was resuspended and centrifuged for 2 hr at 100,000g and 0–2°C through a stepwise sucrose gradient (37, 41, 45, 48%; (w/w). The plasma membrane fraction was collected at the interface between the 37% (d = 1.16) and 41% (d = 1.18) sucrose layer and washed twice with 1 mole/liter $NaHCO_3$. The purity of the membranes was checked by measuring the increase of the specific catalytic activity of 5'-nucleotidase (EC 3.1.3.5) (21) and the decreases of specific activities of succinate dehydrogenase (EC 1.3.99.1) (22) and glucose 6-phosphatase (EC 3.1.3.9) (23), respectively. Labeled membrane associated glycosaminoglycans were isolated and specified as described above for liver cells.

Results. *Synthesis of proteoglycans in embryonic and perinatal hepatocytes.* Embryonic liver contains besides hepatocytes a number of nonparenchymal liver cells (24). Erythroblasts accumulate in embryonic liver but decrease rapidly during the first 5 days of postnatal development (25). To define GAG synthesis in fetal hepatocytes the cells isolated from embryonic liver were incubated together with erythroblasts in presence of [^{35}S]sulfate and thereafter separated from erythroblasts by adhesion to serum-coated plastic petri dishes as described under Materials and Methods. Total GAG synthesis in parenchymal liver cells from embryonic liver compared with that in cells from late postnatal liver is strongly enhanced (Table I). The GAG synthesis rate decreases progressively after birth (Table I). Chondroitin sulfate synthesis in embryonic hepatocytes is about 15 times higher than that measured in parenchymal cells at the sixth day

TABLE I. INCORPORATION OF [^{35}S]SULFATE INTO TOTAL AND SPECIFIC TYPES OF GLYCOSAMINOGLYCANS OF EMBRYONIC AND NEONATAL RAT LIVER HEPATOCYTES

Source of hepatocytes	Total glycosaminoglycans (dpm/mg DNA)	Specific glycosaminoglycans (dpm/mg DNA)	
		Heparan sulfate	Chondroitin sulfate
Embryonic	16160 ± 1000	6300 ± 485	8560 ± 1130
Postnatal			
1 day	9150 ± 860	4120 ± 275	4670 ± 370
2 days	8200 ± 1080	5080 ± 490	3120 ± 410
6 days	5770 ± 780	4900 ± 230	580 ± 120

Note. Hepatocytes were incubated with [^{35}S]sulfate in a mixed cell suspension (together with erythroblasts) for 3 hr and isolated thereafter by adhesion to plastic surfaces. The mean values ± SD of four experiments are given.

of postnatal life. The production of heparan sulfate exhibits only a minor decrease during postnatal development (Table I). The distribution pattern of ^{35}S-labeled GAG in embryonic, postnatal, and adult hepatocytes is shown in Fig. 1. Chondroitin sulfate is synthesized as the major type of GAG in embryonic hepatocytes amounting to more than 60% of total GAG synthesis. Chondroitin sulfate as the major GAG type is replaced by heparan sulfate at the first day of postnatal development (Fig. 1). The synthesis ratio of CS/HS of 1.4 in embryonic liver declines to 0.12 during the first week of postnatal life (Fig. 2). The profile of GAG synthesis of mature hepatocytes is reached at about the 10th postnatal day (Fig. 1).

Erythroblasts, the major type of nonparenchymal cells in fetal liver, synthesized nearly exclusively chondroitin sulfate (98% of ^{35}S-labeled GAG), heparan [^{35}S]sulfate is found only in trace amounts. However, the rate of chondroitin sulfate synthesis of erythroblasts in embryonic tissue is low and contributes less than 10% of total GAG synthesis.

The time-dependent changes of the rate of incorporation of [^{3}H]thymidine into DNA of perinatal liver slices parallel those of chondroitin sulfate synthesis. This is shown by the strong positive correlation ($r = 0.949$) between the synthesis of chondroitin sulfate and the incorporation of [^{3}H]thymidine into DNA (Fig. 3). Because the synthesis of liver heparan

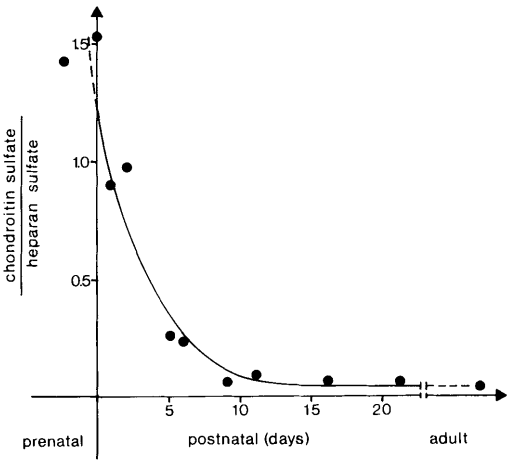

FIG. 2. Developmental changes of the synthesis ratio of chondroitin sulfate to heparan sulfate in isolated hepatocytes.

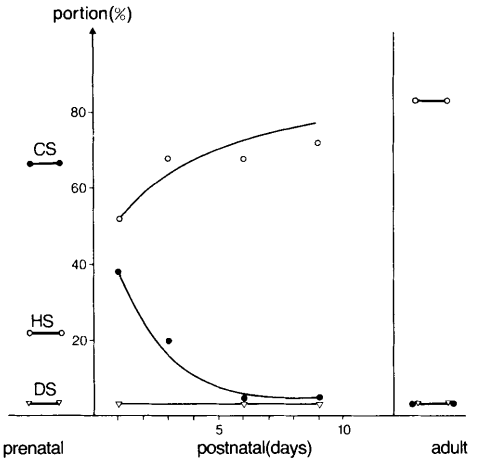

FIG. 1. Relative incorporation of [^{35}S]sulfate into heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) of isolated hepatocytes of late embryonic and early postnatal livers. The hepatocytes were incubated for 3 hr in a mixed cell suspension (together with erythroblasts) and separated thereafter by their adhesion to serum-coated plastic surfaces of petri dishes. Hepatocytes were proteolyzed with papain and ^{35}S-labeled glycosaminoglycans were determined as described under Materials and Methods.

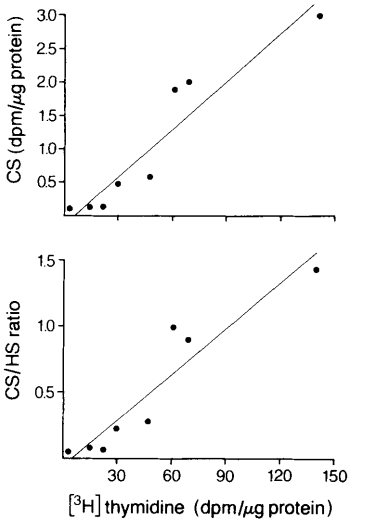

FIG. 3. Correlation between the rates of incorporation of [^{3}H]thymidine into DNA and [^{35}S]sulfate into chondroitin sulfate and the chondroitin sulfate/heparan sulfate ratio, respectively, in late embryonic (20th–22nd day of gestation) and early postnatal ($\leq$10th day of postnatal life) rat livers slices and cells, respectively. The coefficient of correlation is $r = 0.949$ ($n = 8$).

sulfate is almost unchanged during the early development (Table I) the correlation is also found between the chondroitin sulfate/heparan sulfate ratio and [^{3}H]thymidine incorporation into DNA (Fig. 3).

Plasma membrane associated proteoglycans. The pattern of proteoglycans associated with the plasma membranes of early postnatal and adult liver cells was determined in rats injected before with [^{35}S]sulfate. The purity of the membrane preparation is documented by a 12- to 15-fold increase of the specific activity of 5′-nucleotidase and significant reductions of the activity of glucose 6-phosphatase and succinate reductase, respectively (data not shown). Adult liver cell membranes contain almost exclusively heparan sulfate, only traces of ^{35}S-labeled chondroitin sulfate + dermatan sulfate are present in these preparations (Table II). Early postnatal liver cell membranes, however, contain a relatively high portion of chondroitin sulfate. This fraction declines rapidly during the first 10 days of postnatal life and reaches adult levels around the 10th postnatal day (Table II).

Discussion. The data presented in this communication point to the high capacity of fetal hepatocytes to synthesize proteochondroitin sulfate, which was shown to be the major type of GAG produced in these cells. Beginning with birth parenchymal liver cell PCS formation decreases progressively and is lost almost entirely between the 10th and 15th day of postnatal life. It is concluded that the high amounts of PCS in fetal liver (6–8) are produced by the highly stimulated synthesis of this GAG type in the parenchymal liver cells. The assumption is further supported by a strong similarity between the time courses of perinatal changes of the composition of liver GAG (6, 7) and of the GAG synthesis profile in hepatocytes. Both are characterized by a time-related decrease of PCS and dermatan sulfate and an increase of PHS.

A significant portion of newly formed PCS resides in the plasma membrane of the hepatocytes. This fraction declines in parallel to the postnatal shutdown of PCS production, which might be of relevance for some criteria of intercellular communication like cell recognition, membrane transport phenomena, and adhesive properties (7).

The strong positive correlation between the incorporation of [^{35}S]sulfate into PCS and [^{3}H]thymidine into DNA supports the view that this type of proteoglycan might play a role in the regulation of cell proliferation by stimulation of cell division as suggested previously (6–8, 26–30). This is also indicated by the strongly elevated PCS concentration in both experimental (4) and human (3) liver cell carcinoma. Tumorous and embryonic liver tissue exhibit striking similarities in the composition of GAG (5). Our results let us suggest that the increase of PCS in carcinomatous liver is due to its stimulated active synthesis in hepatocellular carcinoma cells by reopening of some fetal pathways of GAG synthesis. A similar mechanism might be active in hepatocytes from chronically injured liver tissue developing fibrosis and cirrhosis, in which PCS becomes a predominant GAG type (1–5). Studies devoted to these problems are currently in progress in our laboratory.

TABLE II. INCORPORATION *in Vivo* OF [^{35}S]SULFATE INTO SPECIFIC TYPES OF GLYCOSAMINOGLYCANS ASSOCIATED WITH PLASMA MEMBRANES FROM ADULT AND POSTNATAL RAT LIVERS, RESPECTIVELY

Source of plasma membranes	Relative distribution of glycosaminoglycans (%)		
	Heparan sulfate	Chondroitin sulfate	Dermatan sulfate
Adult liver	72 ± 1.6	2.7 ± 1.1	
Postnatal liver			
1 day	60 ± 2.0	23.0 ± 4.6	4.0 ± 1.4
2 days	66 ± 3.4	15.5 ± 3.3	—
10 days	64 ± 2.8	5.8 ± 4.2	—

Note. Adult rats received ip 300 μCi, neonatal rats 25 μCi of [^{35}S]sulfate 1 hr before exitus. Plasma membranes were isolated by centrifugation in a discontinuous sucrose gradient. The data represent mean values ± SD of three experiments. In the case of adult liver plasma membranes chondroitin sulfate and dermatan sulfate were not differentiated.

1. Stuhlsatz HW, Vierhaus S, Gressner AM, Greiling H. The distribution pattern and structural differences of the glycosaminoglycans in normal and cirrhotic human liver. In: Popper H, Reutter W, Gudat F, Köttgen E, eds. Structural Carbohydrates in the Liver. Lancaster House Press, Lancaster, Pa., pp542–547, 1982.
2. Murata K, Akashio K, Ochiai Y. Changes in acidic glycosaminoglycan components at different stages of human liver cirrhosis. Hepatogastroenterology **31**: 261–265, 1984.
3. Kojima J, Nakamura N, Kanatani M, Ohmori K. The glycosaminoglycans in human hepatic cancer. Cancer Res **35**:542–547, 1975.
4. Kojima J, Nakamura N, Kanatani M, Akiyama M. Glycosaminoglycans in 3′-methyl-4-dimethylaminoazobenzene-induced rat hepatic cancer. Cancer Res **42**:2857–2860, 1984.
5. Gressner AM. Hepatic proteoglycans—A brief survey of their pathobiochemical implications. Hepatogastroenterology **30**:225–229, 1983.
6. Dietrich CP, Sampaio LO, Toledo OMS, Cássaro CMF. Cell recognition and adhesiveness: A possible biological role for the sulfated mucopolysaccharides. Biochem Biophys Res Commun **75**:329–336, 1977.
7. Sampaio LO, Dietrich CP, Filho OG. Changes in sulfated mucopolysaccharide composition of mammalian tissues during growth and in cancer tissues. Biochim Biophys Acta **498**:123–131, 1977.
8. Sampaio LO, Dietrich CP. Changes of sulfated mucopolysaccharides and mucopolysaccharidases during fetal development. J Biol Chem **256**:9205–9210, 1981.
9. Oldberg A, Höök M, Öbrink B, Pertoft H, Rubin K. Structure and metabolism of rat liver heparan sulphate. Biochem J **164**:75–81, 1977.
10. Prinz R, Klein U, Sudhakaran PR, Sinn W, Ullrich K, von Figura K. Metabolism of sulfated glycosaminoglycans in rat hepatocytes. Synthesis of heparan sulfate and distribution into cellular and extracellular pools. Biochim Biophys Acta **630**:402–413, 1980.
11. Gressner AM, Grouls P. Stimulated synthesis of glycosaminoglycans in suspension cultures of hepatocytes from subacutely injured livers. Digestion **23**:259–264, 1982.
12. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol **13**:29–83, 1976.
13. Devirgiliis LC, Dini L, Di Pierro A, Leoni S, Spagnuolo S, Stefanini S. An improved non-perfusion method for the isolation and purification of rat foetal and neonatal hepatocytes. Cell Mol Biol **27**:687–694, 1981.
14. Gressner AM, Pazen H, Greiling H. The biosynthesis of glycosaminoglycans in normal rat liver and in response to experimental hepatic injury. Hoppe-Seyler's Z Physiol Chem **358**:825–835, 1977.
15. Gressner AM, Heinrigs S, Grouls P. The sequence of changes in the biosynthesis of sulfated glycosaminoglycans in acute, experimental liver disease. J Clin Chem Clin Biochem **20**:15–24, 1982.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
17. Goldberg LR, Parrott DP, Kaplan RS, Fuller GC. Effect of gold sodium thiomalate on proliferation of human rheumatoid synovial cells and on collagen synthesis in tissue culture. Biochem Pharmacol **29**:869–876, 1980.
18. Bachmann W, Harms E, Hassels B, Henninger H, Reutter W. Studies on rat liver plasma membrane altered protein and phospholipid metabolism after injection of D-galactosamine. Biochem J **166**:455–462, 1977.
19. Emmelot P, Bos CJ, Benedetti EL, Rümke P. Studies on plasma membranes. I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. Biochim Biophys Acta **90**:126–145, 1964.
20. Ray TK. A modified method for the isolation of the plasma membrane from rat liver. Biochim Biophys Acta **196**:1–9, 1970.
21. Widnell CC, Unkeless JC. Partial purification of a lipoprotein with 5′-nucleotidase activity from membranes of rat liver cells. Proc Natl Acad Sci USA **61**: 1050–1057, 1968.
22. Pennington RJ. Biochemistry of dystrophic muscle. Mitochondrial succinate tetrazolium reductase and adenosine triphosphatase. Biochem J **80**:649–654, 1961.
23. Baginski E, Foa PP, Zak B. In: Bergmeyer HU, ed. Methoden der enzymatischen Analyse. Weinheim, Verlag Chemie, 3rd ed, pp909–913, 1974.
24. Jones EA. Hepatic sinusoidal cells: New insights and controversies. Hepatology **3**:259–266, 1983.
25. Graf v. Keyserlingk D, Lukow K, Vasel A, Gressner A. Perinatale Änderungen stereologischer Parameter bei der Rattenleber. Anatom Anzeiger **156**:147, 1984.
26. Dietrich CP, de Oca HM. Surface sulfated mucopolysaccharides of primary and permanent mammalian cell lines. Biochem Biophys Res Commun **80**: 805–812, 1978.
27. Takeuchi J. Growth-promoting effect of chondroitin sulphate on solid Ehrlich ascites tumour. Nature (London) **207**:537–538, 1965.
28. Takeuchi J. Effect of intraperitoneal and subcutaneous injection of chondroitin sulphate on the growth of solid Ehrlich ascites tumour. Brit J Cancer **21**:334–337, 1967.
29. Takeuchi J. Effect of chondroitin sulfate on the growth of solid Ehrlich ascites tumor under the influences of other interstitial compounds. Cancer Res **28**:1520–1523, 1968.
30. Takeuchi J. Effect of chondroitinases on the growth of solid Ehrlich ascites tumour. Brit J Cancer **26**:115–119, 1972.

Received April 4, 1985. P.S.E.B.M. 1985, Vol. 180.
Accepted June 25, 1985.