TABLE III. Liver Mucopolysaccharide and Glycoprotein Radioactivity.

Hr after inj of D-glucosamine	% of total bound radioactivity in mucopoly-saccharides	Ratio of specific activity (c/m per μM hexosamine) mucopolysaccharide to glycoprotein		
		.5M fraction	1.5M fraction	2.0M fraction
.5	4.7	1.0	.6	1.9
1	9.3	1.4	3.0	2.7
2	10.5	1.7	3.3	3.3
4	5.2	1.3	2.6	.3
6	3.4	3.2	3.2	.8
8	4.5	.3	1.6	.8
12	2.5	1.2	1.7	.4
48	8.8	3.3	1.3	.6
72	4.2	1.8	1.2	.4

readily by the system synthesizing mucopolysaccharides. The mechanism of incorporation of amino sugar moieties into protein molecules is unknown. The difference in specific activity of the liver glycoproteins and mucopolysaccharides may indicate different mechanisms of incorporation of glucosamine. It is of interest that no chondroitin sulfate or heparin were detected in these rabbit liver preparations. However, Marx *et al.*, have reported a very low heparin concentration (1.11 units/g) in rabbit liver(11).

Summary and conclusion. Studies have been made of the *in vivo* incorporation of radioactive glucosamine into the mucopolysaccharides of rabbit liver. After intraperitoneal injection of radioactive glucosamine, acid mucopolysaccharides with high specific activity were isolated from the liver. Radioactively labeled substances with electrophoretic mobility (on paper) and staining characteristics of hyaluronic acid and heparitin sulfate were found. Glucosamine was the only radioactive substance found in the mucopolysaccharide preparations. This radioactivity amounted to only 2.5 to 10.5% of the total bound radioactivity. A system is apparently present which utilizes preformed glucosamine for the synthesis of acid mucopolysaccharides.

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Received July 3, 1963. P.S.E.B.M., 1963, v114.

Metabolism of Injected $Na_2S^{35}O_4$ in the Rat. I. Effect of Liver Injury on Serum Sulfate.* (28603)

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When inorganic $S^{35}O_4$ is injected intraperitoneally in rats, it rapidly becomes associated with plasma proteins and resists prolonged dialysis even at high pH(1,2,3). All present

* This study was supported in part by USPHS grant.

evidence suggests that this incorporation represents the synthesis of sulfated serum glycoproteins and acid mucopolysaccharides (AMP) and is not due to simple ionic exchange(1,3,4) or to incorporation into the S-S or SH groups of amino acids(13,14). Richmond(1) found that perchloric acid precipitation is a satisfactory way to separate the glycoproteins from other plasma proteins. The perchloric acid soluble glycoproteins contain hexosamine and sulfate but not hexuronic acid; it is, therefore, not an acid mucopolysaccharide.

The site of synthesis of all the serum glycoproteins is not known. Miller and Bale (5), Bostrom *et al.*(6) and Spiro(7) have presented evidence that most serum glycoproteins are synthesized in the liver. Serum glycoprotein concentration has been shown to be depressed in hepatocellular injury(8) and elevated in conditions of rapid or abnormal connective tissue metabolism such as during childhood or in patients with infections, collagen diseases or cancer.

The present investigation is an *in vivo* study of changes in the sulfate metabolism of perchloric acid soluble (PAS) and insoluble (PAI) serum proteins in animals with experimentally induced cirrhosis and hepatomas.

Experimental procedures. The experimental model is the Sprague-Dawley rat maintained on an 8% protein diet containing 0.06% 3'-methyl-4-dimethylaminoazobenzene (3-DAB). This dye will regularly produce a histologic picture of hepatic cirrhosis in 6 weeks and malignant hepatic tumors in 24 weeks; the earliest microscopic changes are seen at 7 days(9).

A total of 74 white adult male rats on the carcinogenic diet were sacrificed after 2, 4, 6, 16, 20 and 24 weeks of treatment. Following intraperitoneal injection of 0.5 mc of $Na_2S^{35}O_4$†, rats were sacrificed in pairs at 1, 2, 4, 8, 12, 24, 48, 76 and 96 hours. Under ether anesthesia each animal was exsanguinated by collecting blood from the inferior vena cava with a dry syringe. The serum was kept frozen at –20°C until just before analysis. The histologic appearance of the liver and the results of the studies were similar in the first 20 weeks of treatment. These 59 animals were grouped together and called the "cirrhotic group." The animals which were sacrificed in the 24th week of the diet all had carcinomas of the liver and were called the "hepatoma group."

An additional 32 animals of the same group maintained on a normal rat chow diet served as controls.

Methods of analysis. Serum was dialyzed across a cellophane membrane for 72 hours against cold running water (3° to 4°C) and for 24 hours against cold (3°C) normal saline. Dialysis of shorter duration did not completely remove added $S^{35}O_4$. When $Na_2S^{35}O_4$ was incubated *in vitro* with normal and abnormal serum or whole blood from 1 to 24 hours at 37°C, dialysis removed all radioactivity. The dialyzed serum was precipitated with 1.8 M perchloric acid by making the dialyzed serum to perchloric acid ratio 1:3. The precipitated perchloric acid insoluble serum proteins (PAI), was oxidized to dryness with a modified Pirie's reagent (10). The dry product was acidified and dissolved in 300 ml distilled water and carrier sulfate as K_2SO_4 containing 2 mg of sulfur was added. This amount of carrier sulfate gave 98 to 101% recoveries and had no effect on self-absorption. After heating to 100°C barium chloride was added slowly in excess and the precipitate allowed to digest overnight. The barium sulfate precipitate was collected on filter paper. This was ignited in a muffle furnace and transferred quantitatively to counting vials containing standard scintillation gel (3 g of 2, 5-diphenyloxazole and 100 mg of 1, 4-di(2, 5-phenyloxazole) benzene and 25 g Thixin‡ per liter of toluene). Counting of radioactivity was accomplished in the Packard tri-carb liquid scintillation spectrometer with automatic sample changer. Duplicate samples were counted at least twice with less than 10% variation in results.

The solution containing the perchloric acid soluble serum proteins (PAS) or seromucoids was treated with 5% phosphotungstic acid in 2 N HCl. The resulting precipitate was dis-

† $H_2S^{35}O_4$ was neutralized to pH 7.0 with NaOH.

‡ Thixin, Baker Castor Oil Co., Bayonne, N. J.

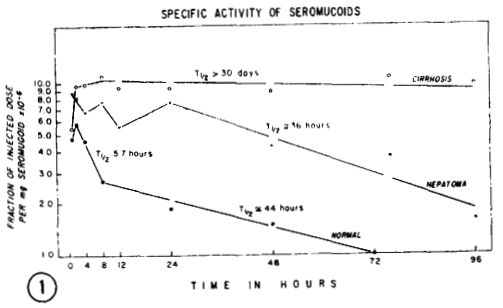

FIG. 1

solved in 0.1 N NaOH and aliquots were analyzed as were the perchloric acid insoluble precipitates. The protein content of the PAI and the PAS precipitates was determined by the biuret method. Radioactivity of dialyzed serum was determined as described above. The fraction of the injected dose of S^{35} per mg of PAI protein and per mg of PAS protein and per mg of dialyzed serum was calculated after counting an appropriately prepared standard together with the samples, thus correcting both for radioactive decay and for any variation of the counting efficiency of the equipment.

Results. Perchloric acid soluble serum proteins (PAS), *i.e.*, seromucoids (Fig. 1): In the 32 animals on a normal diet, $S^{35}O_4$ rapidly associated with the PAS fraction of serum. Peak incorporation occurred within the first 2 hours after intraperitoneal injection. During the period between the 2nd and 8th hour there was a rapid decline of the specific activity having a T 1/2 approximately 5.7 hours, followed by a slower decay with T 1/2 approximately 44 hours.

The pattern of change of the specific activities with time was very different in the 59 animals which had been on the diet from 2 to 20 weeks (cirrhotic group). The peak incorporation still occurred within 2 hours but the subsequent radioactive decay was prolonged with T 1/2 of over 30 days. A much larger fraction of the injected dose of $S^{35}O_4$ was incorporated into a mg of glycoprotein of this group than in normals.

The results in the 15 cancer-bearing rats (hepatoma group), all of which had been on the carcinogenic diet for 24 weeks are more scattered. Rate of decrease of specific activity after 24 hours was similar to that of normals with T 1/2 approximately 36 hours. The specific activity of $S^{35}O_4$ associated with the PAS proteins of this group was greater than that in the normals, but less than in the cirrhotic group. Although the specific activity of the PAS proteins was greater in the cirrhotic group than in the other two, serum concentration of glycoprotein was significantly less (Table I).

Analysis of variance reveals significant differences in the slopes of the curves of the 3 groups.

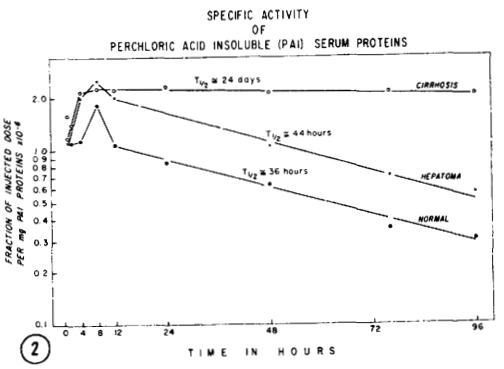

FIG. 2

Perchloric acid insoluble serum proteins (PAI) (Fig. 2): A larger fraction of injected S^{35} was associated with PAI than with PAS because of the large amount of PAI in the serum. The specific activity of PAI, however, was much lower than that of PAS. In general, the specific activities of PAI in the normal animals and in the hepatoma group

TABLE I. Serum Glycoprotein (PAS) Concentration.

Group	Weeks on diet	No. of animals	Mean (mg %)	S.E. of mean	P value*
1. Normal	0	32	292	19.2	
2. Cirrhosis	2–20	59	200	10.3	.001
3. Hepatoma	24	15	282	29.1	.01

* The difference between the means of Groups 1 and 3 is not significant.

were similar. The specific activity of PAI in the cirrhotic group became significantly higher than in the other 2 groups at about 24 hours and remained so throughout the experiment.

Fig. 2 shows the change of specific activities of the PAI with time in the 3 experimental groups. In all three groups peak incorporation occurs at about the 8th hour. In the normal group there is a sharp fall of specific activity between the 8th and 12th hour. After the 12th hour, the decline in specific activity of the PAI describes a straight line ($T\frac{1}{2} = 36$ hours). In the hepatoma group following the 12th hour the $T\frac{1}{2} = 44$ hours. The pattern of change of the specific activities of the cirrhotic group is different from that found in the normal and hepatoma groups. As with the PAS proteins in the cirrhotic group, specific activity declines slowly and beyond the 12th hour $T\frac{1}{2}$ is approximately 24 days.

Discussion. Qualitative changes in serum glycoproteins of patients with acute parenchymal liver injury have previously been documented by Greenspan(11) who demonstrated an increased hexose/protein ratio. Although sulfate has been shown to be associated with the carbohydrate moiety of serum glycoproteins(12), the probably heterogenous nature of these compounds makes a definitive interpretation of patterns of sulfate incorporation impossible. Changes observed in the serum PAI and PAS proteins can therefore only be interpreted as sums of changes occurring within each fraction.

Our findings indicate that major changes in non-dialyzable serum sulfate metabolism occur in both the PAI and PAS fractions in animals developing 3-DAB induced cirrhosis and hepatomas. In normal rats the incorporation of $S^{35}O_4$ per mg PAS proteins was always 2 to 3 times greater than in PAI proteins. In the cirrhotic animals incorporation per mg PAS proteins was always at least 5 times that of PAI proteins. The metabolic breakdown of labeled sulfate-containing serum proteins and subsequent disappearance of radioactivity in both PAS and PAI fractions is remarkably prolonged in our group of cirrhotic rats. Furthermore, the development of hepatomas is associated with changes resulting in the return of the sulfate metabolic pattern toward normal.

Our study of the sulfated serum proteins confirms the presence of quantitative changes in serum glycoproteins in parenchymal liver cell damage. It adds further evidence to confirm profound qualitative changes occurring in both PAS and PAI proteins of serum during hepatic injury.

Summary. The metabolic pattern of S^{35} labelled perchloric acid soluble and insoluble serum proteins was studied following intraperitoneal administration of $S^{35}O_4$ in rats. During the phase of acute liver injury and cirrhosis induced by 8% protein diet containing 0.06% 3-methyl-4-dimethylaminoazobenzene, there was a significant increase in specific activity (fraction of administered dose x 10^{-6}/mg of protein) and a marked prolongation of the half-life time of $S^{35}O_4$ associated with both perchloric acid soluble and insoluble serum protein fractions. With the development of hepatomas the rate of decrease of specific activity of these protein fractions became similar to that of normals. The major portion of the total non-dialyzable S^{35} was associated with the more abundant perchloric acid insoluble serum protein fraction in all animals studied.

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Received January 3, 1963. P.S.E.B.M., 1963, v114.

Response to Nephrotoxic Serum in the Newborn Rat. (28604)

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Intravenous administration of rabbit-anti-rat nephrotoxic serum to newborn rats at 0-3 hours following birth failed to demonstrate at 3 weeks of age or older the typical clinical and biochemical response observed in adult rats(1,2). The response to nephrotoxic serum in adult rats was accompanied by the attachment of the injected heterologous gamma globulin to the kidney antigen in the glomerulus(3). It was also demonstrated that rabbit gamma globulin was attached to the newborn rat glomerulus within 24 hours after intravenous administration of rabbit-anti-rat serum(4). Hammer *et al.*(5) have shown that acute proliferative glomerular changes, associated with proteinuria, occur early in newborn rats following injection with nephrotoxic serum, and that this glomeruuar damage is self-limited by 4 to 6 weeks.

The present study was designed to obtain data not included in our earlier studies during the first and second week periods following administration of nephrotoxic serum in the newborn rat (0 to 3 hours of age). Distinct nephrotoxic effects were found in the kidneys of rats examined at the end of the first week of life with less marked changes at 2 and 3 weeks of age. At 4 weeks and later there were no significant pathologic findings. The plasma biochemical findings at the time when the renal lesion was present did not vary from normal.

Methods. Rabbit-anti-rat nephrotoxic serum was prepared by the method of Smadel (6), modified by Heymann(7). Seventy-one percent of the nephrotoxic serum dose for adult rats was administered intravenously to 27 newborn rats (Wistar strain) within 3 hours after birth, and these animals were sacrificed at 3, 4, 13, 15 and 18 weeks of age. Thirty-seven additional newborn rats were given on a weight basis 4 times the dosage of nephrotoxic serum capable of inducing experimental nephritis in the adult rat and were sacrificed at 1, 2 and 3 weeks of age. One hundred and eighty-six control rats received normal rabbit serum and were sacrificed at 1, 2, 3, 4, 5 and 6 weeks of age. The day before sacrifice, each animal 3 weeks and older was placed in a metabolic cage with water *ad libitum* and timed urine specimens were collected for protein analysis.

Sera obtained from rats of the same age and experimental design were pooled and biochemical analyses were done in duplicate. Renal tissue obtained at autopsy was fixed in formaldehyde and paraffin sections were stained with hematoxyin and eosin and with the PAS reagent. Urea nitrogen was determined by the micro-diffusion method of Conway(8), and cholesterol by the method of Bloor(9). Serum protein electrophoresis was analyzed with a Spinco apparatus. Urine proteins were determined by the method of Hiller, McIntosh and Van Slyke(10), and serum proteins by the method of Gornall, Bardawill and David(11).

Results. 1. *Weight:* The data are presented in Tables I, II and III. Animals sacrificed at 1 week of age did not show significant difference in mean weight between controls and those injected with 4 times the nephrotoxic dose, 10.6 ± 1.2 g *vs* 11 ± 2.8

* Aided by USPHS grants.

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