Serum and Tissue Glycoproteins in Mice Bearing Transplantable Tumors.* (18151)

H. R. CATCHPOLE. (Introduced by G. A. Bennett.)

From the Department of Pathology, University of Illinois, College of Medicine.

It has long been known that protein-bound carbohydrate increases in the blood of cancer patients(1). More recently, Seibert *et al.* (2) observed a rise in serum polysaccharide and in α_2 globulin in tuberculosis, sarcoidosis and carcinoma; they attributed these increases to tissue destruction. Winzler *et al.*(3) and Winzler and Smyth(4) found an increase in the mucoprotein fraction of cancer and pneumonia sera, which they attributed to an abnormality in protein metabolism. Shetlar *et al.*(5) studied a serum polysaccharide component which increased following inflammation. They postulated that this rise was associated with tissue proliferation and repair. Recent studies(6) suggested that increased circulating sugar-containing proteins might arise from a connective tissue component or components of a glycoprotein nature. Such components may be visualized histochemically by the McManus-Hotchkiss periodic acid leucofuchsin technic, and can be demonstrated normally to be water and alcohol insoluble. Following traumatic injury to tissue and also after induced lung edema, the local state of the ground substance of the connective tissue changed, and it became water soluble. This change was ascribed to the secretion of depolymerizing enzymes which produced breakdown of a highly polymerized, insoluble ground substance to less highly polymerized residues. It was postulated that the latter might be soluble and diffusible, and appear as glycoprotein components of serum. Part of the evidence for the above thesis rested on the finding, in the region of fast growing tumors, of jelly-like, easily ruptured connective tissue strands, in place of the coherent, resistant connective tissue normally encountered, and on the demonstration of water solubility of the ground substance of this tissue in histological sections.

Materials and methods. Young adult Swiss and C_3H male mice were used. They received implants, into the abdominal subcutaneous region, of the fibrosarcomas: Earle HGW, OA and L (obtained through the courtesy of Dr. Thelma Dunn, National Institute of Cancer). After varying lengths of time, small (2-3 mm), large (1.0 cm), and occasionally very large (over 2.0 cm) tumors were found. Usually 4 or more animals were taken in groups of comparable tumor size, anesthetized, and bled from the right heart. Connective tissue from the tumor site was collected by means of fine scissors and forceps, transferred immediately to a chilled tube and the pooled material lyophilized. Blood and subcutaneous connective tissue were also obtained from groups of normal mice; in these, the skin was pinned out and connective tissue stripped off by fine toothed forceps. For determining plasma glycoproteins the method of Winzler *et al.*(3) was used.† Plasma was diluted 1:1 with water and plasma proteins precipitated by the addition of 2 volumes of 0.75M perchloric acid; from the filtrate, glycoproteins were precipitated with ½ volume of 5% phosphotungstic acid in 2N HCl. Gly-

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† We are greatly indebted to Dr. R. J. Winzler for supplying details of their methods antecedent to publication.

TABLE I. Plasma Mucoproteins in Normal Mice and Mice Bearing Transplantable Tumors.

	No. of animals	Sugar as galactose-mannose, mg/100 cc	Tyrosine, mg/100 cc	Sugar/ tyrosine
Normal Swiss	40	16.9 ± 1.95*	3.96 ± 0.6*	4.05
Tumor bearing Swiss				
Earle HGW—large	5	28.0	7.35	3.80
medium	3	18.4	6.15	3.00
small	16	16.4	4.30	3.85
Normal C_3H	6	17.7	3.8	4.65
Tumor bearing C_3H				
Earle HGW—large	5	63.5	5.78	11.0
OA—large	6	57.6	5.45	10.6
L—large	8	34.7	7.05	4.9
L—small	5	19.0	5.4	3.5

* Stand. error of mean.

TABLE II. Mucoproteins of Subcutaneous Connective Tissues of Normal Mice and of Connective Tissue Associated with Subcutaneously Transplanted Tumors.

	No. of animals	Sugar as galactose-mannose, mg/100 g	Tyrosine, mg/100 g	Sugar/ tyrosine
Normal Swiss	50	230 ± 37.5*	26.6 ± 4.5*	8.6
Tumor bearing Swiss				
Earle HGW—large	5	300	50.0	6.0
medium	3	275	55.0	5.0
Normal C_3H	6	181	19.2	9.5
Tumor bearing C_3H				
Earle HGW	5	565	62.5	9.0
OA	6	—	29.5	—
L—large	8	305	23.5	12.8
L—small	5	392	28.5	13.8
L—very large	1	400	92.0	4.4

* Stand. error of mean.

coprotein was estimated by the use of two methods: (1) sugar with the orcinol reagent, against a galactose-mannose standard; (2) protein with the phenol reagent of Folin and Ciocalteu, against a standard of tyrosine.

The dried connective tissue samples were ground with methanol-chloroform (1:1) in a small mortar. The solvent was removed by centrifugalization and replaced successively by acetone and ether. Finally the defatted residue was incubated overnight with absolute alcohol to effect denaturation. The alcohol was removed and the connective tissue dried and weighed. It was triturated with a small quantity of phosphate buffer (1.0-2.0 ml) at pH 7.0 for 30 minutes, after which the tissue fragments were removed by brief centrifugalization at 18000 G. The supernatant solution was removed with a syringe and thereafter treated similarly to the sample of diluted blood plasma in the Winzler procedure.

Results. Enhanced amounts of plasma glycoproteins were readily shown in the presence of large tumors. With practical uniformity, values for glycoproteins whether determined as carbohydrate or as protein were increased in both strains of mice and for all tumors studied (Table I). The individual sugar/tyrosine ratios were somewhat variable, but the means (normal, 4.2; tumor, 4.1) essentially agree with the values found in the human by Winzler *et al.*(3) (normal, 3.69 ± 0.23; cancer, 3.85 ± 0.30). Partly these variations may be explained by difficulties of blood collection and determination of small samples. In two series, the plasma glycoprotein values were relatively little increased for small and medium sized tumors and greatly increased for very large tumors. That agreement may not always be absolute between tumor size and carbohydrate release may be due to the unpredictable biological

status of any tumor of a given size.

Water soluble glycoproteins of tumor connective tissue show distinct increases as compared with normal connective tissue in Swiss and C_3H mice implanted with various tumors (Table II). Both sugar and protein (tyrosine) values are elevated. In some cases the sugar/tyrosine ratios show wide fluctuations from the expected values; the reason for this has not been explained.

Summary and conclusions. Serum glycoproteins are increased in mice bearing transplantable tumors. Water soluble glycoproteins are increased in the connective tissue bordering and abutting on the tumor. These findings support the thesis that increased circulating glycoproteins arise from the ground substance of the connective tissue at the site of invasive growth by a process of depolymerization, whereby smaller, water soluble and diffusible glycoprotein moieties are produced.

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Quantitative Estimation of Plasma Accelerator-Globulin. (18152)

JOHN R. CARTER AND E. D. WARNER.

From the Department of Pathology, College of Medicine, State University of Iowa, Iowa City.

In a previous publication(1), a method was described whereby oxalated bovine plasma could be rendered free from Ac-globulin. This solution ($HgCl_2$-plasma), containing 50-60 Iowa units/ml of prothrombin, has provided a stable, reliable source of prothrombin for the quantitative estimation of Ac-globulin in plasma. The method of assay, based upon the use of three variables—conversion rate, thrombin yield, and onset of thrombin formation, is a modification of the method of Ware and Seegers(2).

Method. 1. Human and dog whole blood is collected in 1.85% potassium oxalate; 7 parts of blood to 1 part of anticoagulant are used. For rats, 1 ml of blood is taken into a syringe containing 0.23 ml of 1.85% potassium oxalate. The blood is centrifuged at 5000 r.p.m. for 15 minutes, and the hematocrit and oxalate correction factor recorded.

2. Determine the amount of prothrombin in the plasma to be tested for Ac-globulin using the modified two-stage technic(3,4).

3. Into a chemically clean glass test tube (120 x 13 mm), place 4 ml of physiologic saline.

4. Add $HgCl_2$-plasma in an amount such that the prothrombin in the $HgCl_2$-plasma, plus the amount of prothrombin in the plasma to be assayed for Ac-globulin equals 50 units.

5. To the diluted $HgCl_2$-plasma, add 0.08 ml of the human plasma (0.008 ml for dog, rat, and beef plasma) to be assayed.

6. Add physiologic saline to the tube to bring the total volume to 5 ml. The prothrombin concentration is now 10 units/ml. Swirl tube to insure thorough mixing of components.

7. To 1.5 ml of incubation mixture,* add 0.5 ml of contents of the dilution tube (step 6).

8. Incubate at 28°C for 5, 8, 11, 14, 17, 23, 26, and 30 minutes. At the end of each of these time periods, add 0.2 ml of the contents of the incubation tube (step 7) to 0.05 ml of a 1.0% solution of Armour bovine fibrinogen, and record the clotting time.

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* Incubation mixture is prepared as follows: calcium saline (1% $CaCl_2$ in physiologic saline), 3 parts; 15% acacia (containing 0.04% calcium by weight), 2 parts; imidazole buffer (pH 7.2), 1 part. To 10 ml of this solution is added 5 ml of heated crude lung thromboplastin.